University of Alberta

New Antimicrobial Agents Acting on Bacterial Cell Walls

by

Sylvie Garneau ©

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of the requirements for the degree of
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ABSTRACT

Two approaches were undertaken to study potential inhibitors of peptidoglycan biosynthesis. Three types of compounds were designed as potential inhibitors of glycosyltransferase, a key enzyme for the formation of bacterial cell wall. Syntheses of α-*C*-glycosides and disaccharide analogs of lipid II were unsuccessful. Target *O*-(dimethyl (*Z*)-2-oxymethyl-3-octylbutenedioate) 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-*D*-glucopyranoside (**81**) was prepared by coupling dimethyl (*Z*)-2-hydroxymethyl-3-octylbutenedioate (**73**) and phenyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-tetrachlorophthalimido-1-thio-β-*D*-glucopyranoside (**52**) (4 steps, 36% overall yield). (*Z*)-2-(2-Acetamido-2-deoxy-α-*D*-glucopyranosyl)oxymethyl-3-tetradecylbutenedioic acid dilithium salt (**98**) was synthesized from 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-*D*-glucopyranosyltrichloroacetimidate (**85**) and dimethyl (*Z*)-2-hydroxymethyl-3-tetradecylbutenedioate (**74**) (7 steps, 22% overall yield). Chaetomellic acid A dilithium salt (**13**), (*Z*)-2-geranyl-3-methylbutenedioic acid dilithium salt (**14**), (*Z*)-2-farnesyl-3-methylbutenedioic acid dilithium salt (**15**) and (*Z*)-2-nerolyl-3-methylbutenedioic acid dilithium salt (**16**) were prepared by conjugate addition to dimethyl acetylenediacarboxylate followed by ester hydrolysis (72-81% overall yield). Compounds **13-16** were tested as inhibitors for rubber transferases from *Hevea brasiliensis* and *Parthenium argentatum*. No inhibition was observed for compounds **14** and **16**. Compound **13** is a competitive inhibitor of *H. brasiliensis* and *P. argentatum* (K_i = 42 μM and 8.8 μM, respectively). Compound **15** is a competitive inhibitor of *P. argentatum* (K_i = 25 μM) and a non-competitive inhibitor of *H. brasiliensis* (K_i = 140 μM).

Bacteriocins were also studied as potential inhibitor of peptidoglycan biosynthesis. Piscicolin 126, carnobacteriocins B1 and BM1, and a new bacteriocin were isolated from *Carnobacterium piscicola* 307 and 682, characterized by mass spectrometry and amino acid sequencing. *Brochothrix campestris* ATCC 43754 produces a two-component bacteriocin, brochocin C (BrcC). An improved purification method was developed for BrcC using *n*-butanol and chloroform extraction. Mass spectral characterization of the two components, BrcA and BrcB, showed that both are excreted into the medium by *B. campestris* as mature peptides (59 and 43 amino acids, respectively). Separate expression clones of both peptides had been constructed in *C. piscicola* LV17C, but the products had not been chemically characterized. Purification by the new protocol showed that BrcA is expressed as the mature peptide, but that BrcB is produced by *C. piscicola* as a fragment, BrcB(10-43), which has been cleaved at an internal Gly-Gly site. In combination with BrcA, this fragment displays the full activity of the BrcC complex. Circular dichroism measurements revealed a high β-sheet content in the secondary structure of both peptides, as well as in a 1:1/BrcA:BrcB(10-43) mixture. Separate expression clones of BrcA and BrcB were constructed in *E. coli*, but these produced multiple fragments of the desired peptides with little or no activity. Various culture media (peptone from blue-green algae, Celtone®-U and SASM) were also experimented in hope of labeling BrcA and BrcB, but peptides with poor activity were recovered after growth. A maltose-binding protein fusion of BrcA and an intein protein fusion of BrcB in *E. coli* were constructed to produce large quantities of labeled peptides for structure elucidation by NMR. Studies on these fusion systems are currently underway.

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LIST OF ABBREVIATIONS

$[\alpha]_D^{26}$	specific rotation
A or Ala	alanine
ABC	ATP-binding cassette
Abu	aminobutyric acid
Abu-S-Ala	β-methyllanthionine
Ac	acetyl
Ac_2O	acetic anhydride
AcOH	acetic acid
Anal.	analysis
Ala-S-Ala	lanthionine
APT	attached proton test
aq.	aqueous
Bn	benzyl
bp	boiling point
br	broad
BrcA	brochocin A
BrcB	brochocin B
BrcC	brochocin C
c	concentration
C or Cys	cysteine
Calcd	calculated

CAN	ceric ammonium nitrate
CBD	chitin-binding domain
CbnB1	carnobacteriocin B1
CbnB2	carnobacteriocin B2
CbnBM1	carnobacteriocin BM1
CD	circular dichroism
Chp	chlorohydroxyphenylglycine
CoA	coenzyme A
COSY	correlation spectroscopy
CsCl	cesium chloride
δ	chemical shift in parts per million downfield from tetramethylsilane
d	doublet
D or Asp	aspartic acid
Da	Dalton
m-DAP	*meso*-diaminopimelic acid
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
Dha	dehydroalanine
Dhb	dehydrobutyrine
DHP	dihydropyran
DMAD	dimethyl acetylenedicarboxylate
DMAP	4-(dimethylamino)pyridine
DMAPP	dimethylallyl diphosphate
DMF	dimethylformamide

DMSO	dimethylsulfoxide
DMTST	dimethyl-(methylthio)sulfonium triflate
dNTP	mixture of dATP, dCTP, dGTP, dTTP (2'-deoxynucleoside 5'-triphosphates)
DTT	dithiothreitol
E or Glu	glutamic acid
EI	electron impact
eq	equivalent
ES	electrospray
Et	ethyl
EtBr	ethidium bromide
Et_2O	diethyl ether
EtOH	ethanol
F or Phe	phenylalanine
FAB	fast atom bombardment
FPP	farnesyl diphosphate
G or Gly	glycine
Glc	glucose
GlcNAc	*N*-acetylglucosamine
GTase	glycosyltransferase
H or His	histidine
HMPA	hexamethylphosphoramide
HMQC	heteronuclear multiple quantum coherence

HPLC	high performance liquid chromatography
HRMS	high-resolution mass spectrum
I or Ile	isoleucine
IPA	isopropyl alcohol
IPTG	isopropyl-β-D-thiogalactopyranoside
IR	infrared
J	coupling constant
K or Lys	lysine
KOAc	potassium acetate
L or Leu	leucine
LAB	lactic acid bacteria
LeuA	leucocin A
lit.	literature reference
m	multiplet
M or Met	methionine
m/*z*	mass to charge ratio
MALDI-TOF	matrix-assisted laser desorption / ionization time-of-flight
MBP	maltose-binding protein
m-CPBA	*m*-chloroperoxybenzoic acid
MCS	multiple cloning site
Me	methyl
MeCN	acetonitrile
MeOH	methanol

MGT	non-penicillin-binding monofunctional glycosyltransferase
min	minute(s)
mp	melting point
MurNAc	*N*-acetylmuramic acid
MW	molecular weight
N or Asn	asparagine
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NaOAc	sodium acetate
NaOH	sodium hydroxide
NaOMe	sodium methoxide
Na_2SO_4	sodium sulfate
NBS	*N*-bromosuccinimide
n-BuOH	*n*-butanol
NIS	*N*-iodosuccinimide
NMR	nuclear magnetic resonance
nm	nanometers
N-PSP	*N*-phenylselenophthalimide
NOE	nuclear overhauser effect
ORF	open reading frame
P or Pro	proline
PBP	penicillin-binding protein
PCR	polymerase chain reaction
PFTase	protein farnesyltransferase

PGM	peptidoglycan monomer
Phth	phthaloyl
P_i	phosphate
PMB	*p*-methoxybenzyl
PP_i	pyrophosphate
ppm	parts per million
PPTS	pyridinium *p*-toluene sulfonate
PST	phenylsulfenyltriflate
pyr	pyridine
q	quartet
Q or Gln	glutamine
quant.	quantitative yield
qn	quintet
R or Arg	arginine
R_f	retention factor
rt	room temperature
S or Ser	serine
SDS	sodium dodecyl sulfate
SEM	trimethylsilylethoxymethyl
$SnCl_2$	tin chloride
t	triplet
T or Thr	threonine
TBE	tris-borate-EDTA

TCP	tetrachlorophthaloyl
Tf	trifluoromethanesulfonyl
TFA	trifluoroacetic acid
TFE	trifluoroethanol
THF	tetrahydrofuran
THP	tetrahydropyran
TLC	thin layer chromatography
TMSOTf	trimethylsilyl trifluoromethanesulfonate
TPase	transpeptidase
t_R	retention time
Tris	tris-(hydroxymethyl)aminomethane
Ts	*p*-toluenesulfonyl
p-TsOH	*p*-toluenesulfonic acid
UDP	uridine diphosphate
UMP	uridine monophosphate
UTP	uridine triphosphate
V or Val	valine
W or Trp	tryptophan
WRP	washed rubber particles
Xgal	5-bromo-4-chloro-3-indolyl-β-*D*-galactose
Y or Tyr	tyrosine

CHAPTER 1. Inhibition of Peptidoglycan Biosynthesis

INTRODUCTION

The introduction of antibiotics for the treatment of bacterial infections is one of the greatest medical achievements of the past century. However, through gene exchange and mutation, some life-threatening bacterial species (*Enterococcus faecalis*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*) have become resistant to all known antibiotics.[1-3] The first example of antibiotic resistance was observed for *Staphylococcus aureus* in the early 1940s, shortly after the discovery of penicillin.[4] Nowadays, the widespread occurrence of acquired bacterial resistance to antibiotics constitutes a serious threat to global public health[5-9] and necessitates the development of new antimicrobial agents that circumvent known resistance mechanisms including:[10-14]

1. Substrate deactivation by enzymatic modification
2. Target modification and/or over-expression, thereby reducing or eliminating the binding of the drug
3. Modified cellular uptake (active efflux or reduced uptake), thereby preventing the antibiotic from reaching its target

The molecular basis of clinically useful antimicrobial agent action is well documented (Table 1) and provides guidance for the discovery of new antibiotics. As a result of the importance of the cell envelope for the viability of bacteria, antibiotics that interfere with its function and biosynthesis represent a primary target for drug development. Since bacteria are most vulnerable to inhibition of the peptidoglycan biosynthetic pathway that is non-existent in mammals, numerous new potential antibacterial agents are targeted towards this physiological process.

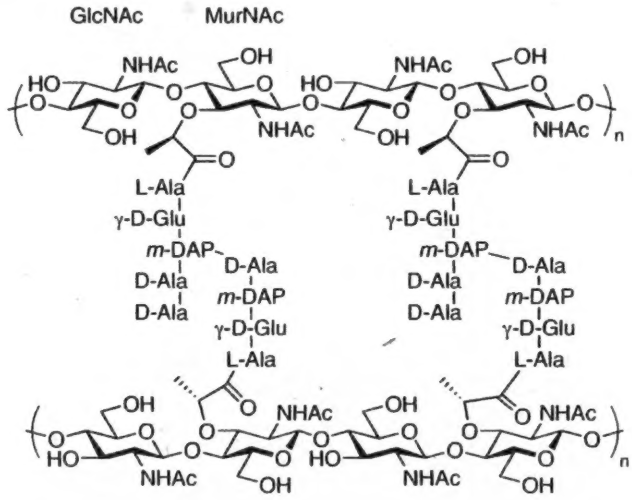
Table 1. Mode of action of most widely used antibiotics

Function inhibited	***Class$^{Ref.}$***	***Drug$^{Ref.}$***	***Molecular Target***
Peptidoglycan biosynthesis		Bacitracin[15-17]	Undecaprenyl pyrophosphate (translocation across membrane)
		D-Cycloserine[18]	D-Ala racemase D-Ala-D-Ala ligase
		Fosfomycin[19-25]	UDP-GlcNAc enolpyruvyl transferase
	β-Lactams[26]	Cephalosporins[27] Penicillins	Transpeptidases Carboxypeptidases
	Glycopeptides	Vancomycin[28,29] Teicoplanin	Peptidyl D-Ala-D-Ala (cell wall peptidoglycan)
Protein synthesis		Chloramphenicol[30-33]	50S ribosomal subunit
		Fusidic acid[34-37]	Elongation factor G
	Aminoglycosides		30S ribosomal subunit
	Macrolides		50S ribosomal subunit
	Oxazolidinones	Zyvox[38-41]	50S ribosomal subunit
	Streptogramins		50S ribosomal subunit
	Tetracyclines		30S ribosomal subunit
DNA replication/transcription	Quinolones[39,42-44]	Ciprofloxacin	Gyrase and topoisomerase IV
Transcription	Rifamycins[45,46]		RNA polymerase
Folate synthesis	Sulphonamides[47]		Dihydropteroate synthetase

1. Peptidoglycan

An essential feature in the life cycle of both Gram-positive and Gram-negative bacteria is the formation of new cell wall. Peptidoglycan, also called murein, a single covalent polymeric macromolecule found on the outside of the cytoplasmic membrane of almost all eubacteria, is the major and the most important structural constituent for the survival of the bacterial cell. Its main functions are to preserve a defined cell shape and to maintain cell integrity against high internal osmotic pressure by providing strength and rigidity to the cells.[48-53] The peptidoglycan layer consists of a matrix of repeating disaccharide units cross-linked through pentapeptide side chains[48,54,55] (Figure 1). The polysaccharide backbone is composed of alternating β-1,4-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc). The short peptide chains, appended to the muramyl moiety, have for general sequence L-Ala-γ-D-Glu-**X**-D-Ala-D-Ala, where **X** is an L-amino acid containing an amino group in the side chain. In Gram-negative bacteria **X** is *meso*-diaminopimelic acid[56,57] (*m*-DAP), whereas in Gram-positive bacteria **X** is usually L-lysine.[58] Cross-linking of neighboring glycan chains occurs either by a direct peptide bond or by a short peptide bridge between two peptide subunits.[54,59] The degree of interlinking that provides structural rigidity varies from 25-50% and 70-90% in Gram-negative and Gram-positive bacteria, respectively.[60]

Figure 1. Structure of the peptidoglycan layer in Gram-negative bacteria

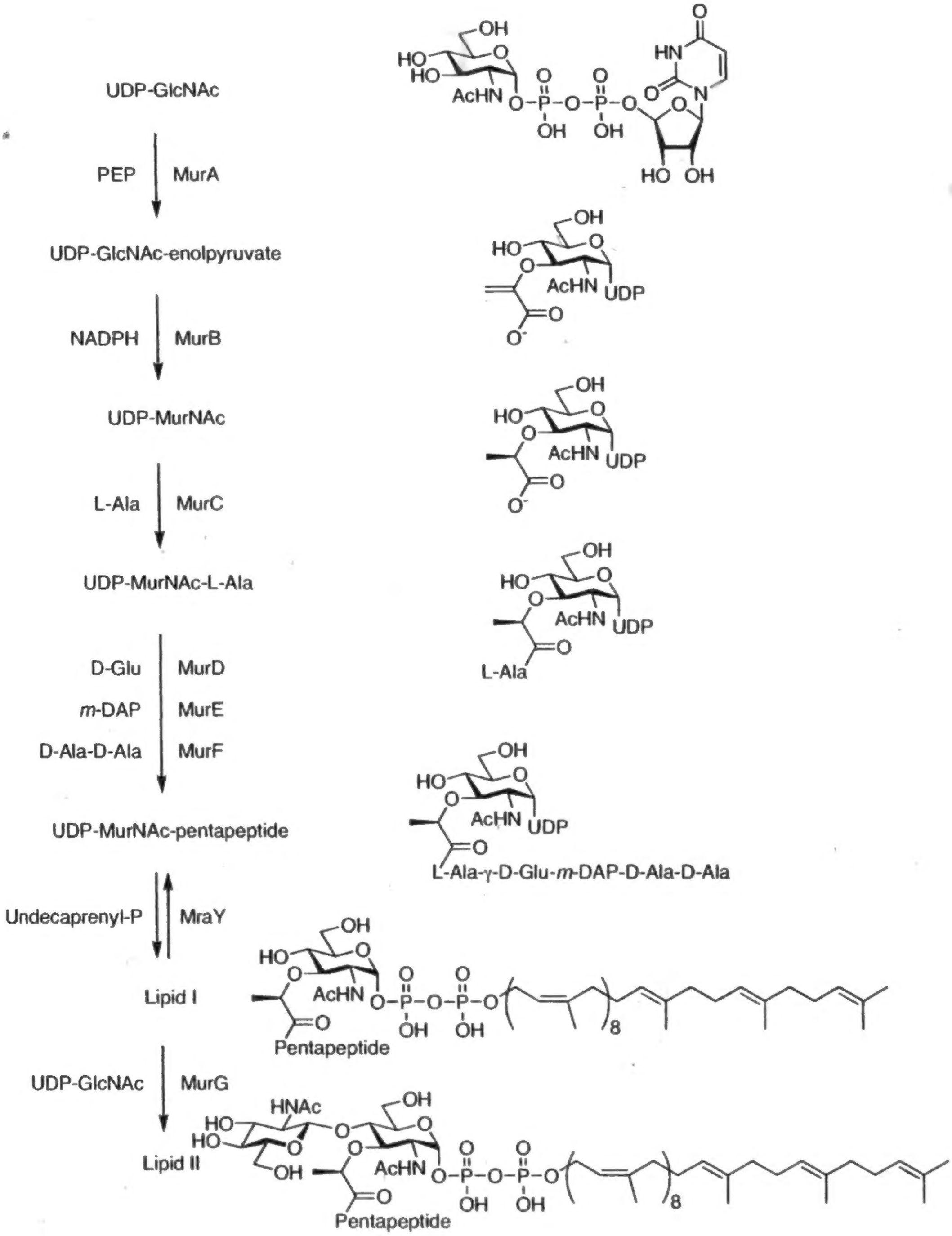


1.1. Peptidoglycan Biosynthesis

Recently the biosynthesis of peptidoglycan has been greatly reviewed.[12,61-64] The first stage of this complex two-stage process consists of the assembly of the peptidoglycan monomer (PGM or lipid II) units in the cytoplasm or at the inner side of the cytoplasmic membrane (Figure 2). The well-defined pathway from UDP-*N*-acetylglucosamine (UDP-GlcNAc) to the final lipid intermediate proceeds *via* a series of eight enzyme-catalyzed reactions. The genetic and physiological data for these enzymes have also been studied and reviewed.[61,65,66] The crystal structures of *Escherichia coli* MurF[67] and MurG[68] have been reported.

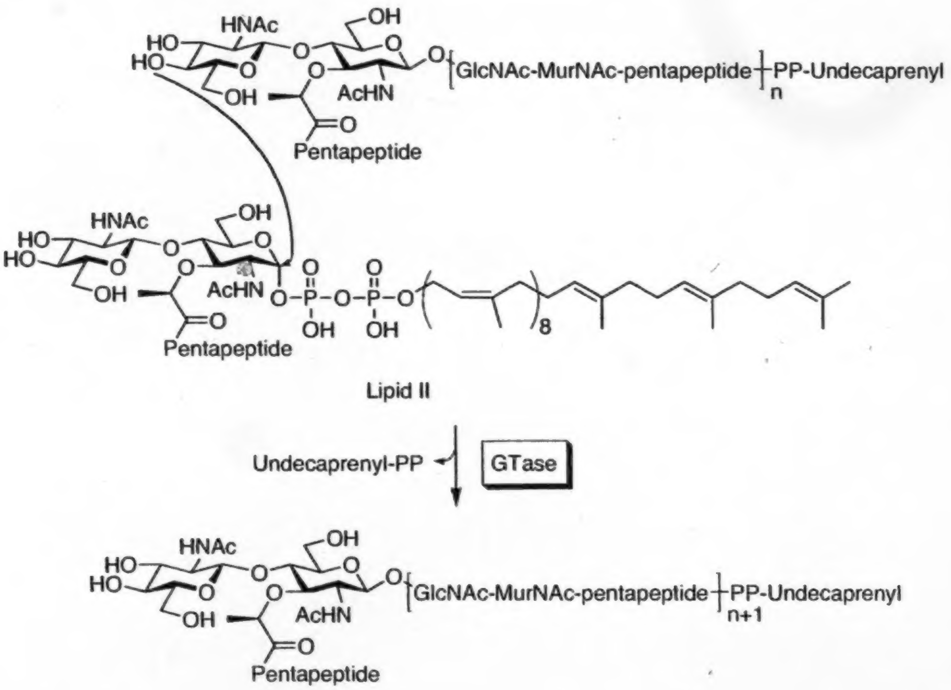
The first step of the peptidoglycan biosynthesis involves the transfer of an enolpyruvyl group from phosphoenolpyruvate (PEP) to UDP-GlcNAc. Reduction of the resulting unsaturated acid by NADPH leads to the formation of UDP-*N*-acetylmuramic acid (UDP-MurNAc). At this point a stepwise addition to the lactic acid residue of UDP-MurNAc of three amino acid residues (L-Ala, D-Glu and *m*-DAP), followed by the addition of the preformed D-Ala-D-Ala dipeptide, completes the synthesis of the precursor UDP-MurNAc-pentapeptide. In the next step, UDP-MurNAc-pentapeptide is transferred to undecaprenyl phosphate, a carrier molecule found in the cytoplasmic membrane. A second GlcNAc moiety is then coupled at the 4-position of lipid I by a glycosylation reaction catalyzed by *N*-acetylglucosaminyl transferase MurG to form the final lipid intermediate, lipid II. Total syntheses of lipid I[69-71] and lipid II[72,73] have been reported.

Figure 2. Stepwise assembly of lipid II


UDP-GlcNAc
PEP
MurA
UDP-GlcNAc-enolpyruvate
NADPH
MurB
UDP-MurNAc
L-Ala
MurC
UDP-MurNAc-L-Ala
D-Glu
MurD
m-DAP
MurE
D-Ala-D-Ala
MurF
UDP-MurNAc-pentapeptide
L-Ala-γ-D-Glu-m-DAP-D-Ala-D-Ala
Undecaprenyl-P
MraY
Lipid I
Pentapeptide
UDP-GlcNAc
MurG
Lipid II
Pentapeptide
AcHN
HNAc
UDP
8

The second stage of the biosynthesis of peptidoglycan involves translocation of the PGM through the cytoplasmic membrane and polymerization reactions on the outer surface of the membrane. Two types of enzymes are involved in the polymerization process: glycosyltransferase (transglycosylase) (GTase) and transpeptidase (TPase). GTase catalyzes the formation of new linear glycan strands by creation of a β-1,4-glycosidic linkage between a MurNAc residue of lipid II and the terminal GlcNAc residue of the growing polysaccharide chain (Figure 3).[63] Undecaprenyl pyrophosphate is released during the transglycosylation and recycled *via* dephosphorylation to undecaprenyl phosphate that is to be reused in the formation of lipid II. TPase catalyzes the cross-linkage between the new chains and the binding of the nascent peptidoglycan to the preexisting cell wall.[74-76]

Figure 3. Transglycosylation reaction


HNAc
OH
HO
HO
O
OH
AcHN
O
Pentapeptide
GlcNAc-MurNAc-pentapeptide
PP-Undecaprenyl
n
P
OH
8
Lipid II
Undecaprenyl-PP
GTase
n+1

2. Glycosyltransferase (GTase)

Of all the enzymes involved in peptidoglycan biosynthesis GTase is probably the most interesting target for antibiotic development. Located on the cell surface GTase is easily accessible to small molecular drugs. New antimicrobial agents that interfere with GTase may therefore be less prone to resistance development.

2.1. GTase Activity

The ability to directly assess GTase activity depends on the precise form of the lipid II substrate and exact reaction conditions.[76] Activity against GTase can be detected by a method described by van Heijenoort and co-workers.[77] Several enzymes are responsible for GTase activity. A number of bifunctional enzymes, known as class A penicillin-binding proteins (PBPs), are involved in transglycosylation and transpeptidation. Systematic studies on *Escherichia coli* mutants lacking all possible combinations of eight PBPs[78] confirmed that PBP1A and PBP1B[79,80] are required for the survival of the bacterial cell.[81,82] These high-molecular-weight PBPs, located in the periplasm, are anchored to the cytoplasmic membrane *via* N-terminal hydrophobic signal sequences.[83] Their GTase activity resides in the N-terminal half of the protein.[84] PBP1B can be purified from *E. coli* by a moenomycin-mediated affinity purification technique described by Stembera *et al.*[85] PBP1C of *E. coli*, a close homolog to the bifunctional TPases/GTases PBP1A and PBP1B, has been found to function *in vivo* as a GTase only.[86] PBP2a of *Streptococcus pneumoniae* has also been reported as a major GTase and valuable target for the discovery of new antimicrobial agents.[87] It has been proposed that PBP2a interacts with the cytoplasmic membrane in a region distinct from its transmembrane anchor, which is located in the GTase domain.[88] Studies of PBPs 1a, 1b and 2a in *S. pneumoniae* showed that PBP1a and PBP2a are essential for the viability of the bacteria.[89,90]

In addition to the PBPs, a variety of membrane bound, non-penicillin-binding, monofunctional GTase (MGT) capable of catalyzing the formation of uncross-linked peptidoglycan are found in *E. coli*,[91] *M. luteus*,[92] *S. aureus*,[92,93] and *S. pneumoniae*.[94] Other genes encoding MGT have been detected on the basis of sequence homology in a variety of bacterial strains (*Escherichia coli, Haemophilus influenzae, Klebsielle pneumoniae, Neisseria gonorrhoeae, Ralstonia eutropha*, and *Staphylococcus aureus*).[95-97]

2.2. Natural Inhibitors of the Transglycosylation Reaction

Natural compounds that inhibit the transglycosylation reaction are divided into two categories:

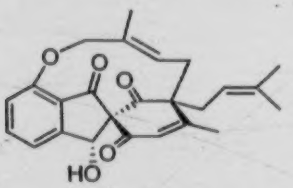
1. Compounds that bind to lipid II (*e.g.* Bacteriocins of lactic acid bacteria will be discussed and studied in Chapter 2)
2. Products that directly inhibit the enzymes (phosphoglycolipids produced by various species of *Streptomyces*.[98] *e.g.* moenomycin)

2.2.1. Compounds that Bind to Lipid II

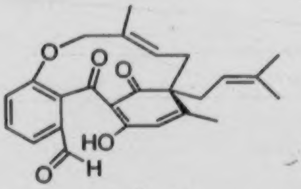
In the past few years, naturally occurring novel inhibitors of GTase such as coleophomone A (**1**) and B (**2**),[99] and cyclic depsipeptide antibiotics katanosin B (**3**) and plusbacin A_3 (**4**)[100] have been isolated (Figure 4). The mode of action of compounds **3** and

4 is not yet precisely understood. However, their inhibition seems to result from binding to the lipid intermediate rather than to the enzyme directly.

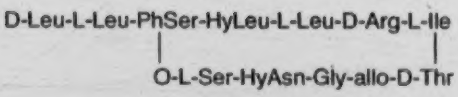
Figure 4. Structure of novel naturally occurring inhibitors of GTase



1

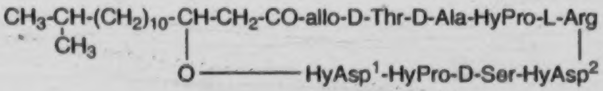


2



$$\begin{array}{l}\text{D-Leu-L-Leu-PhSer-HyLeu-L-Leu-D-Arg-L-Ile}\\ \qquad\quad | \qquad\qquad\qquad\qquad\qquad\qquad\quad |\\ \qquad\quad \text{O-L-Ser-HyAsn-Gly-allo-D-Thr}\end{array}$$

3 PhSer = L-*threo*-β-phenylserine
HyLeu = L-*threo*-β-hydroxyleucine
HyAsn = L-*threo*-β-hydroxyasparagine

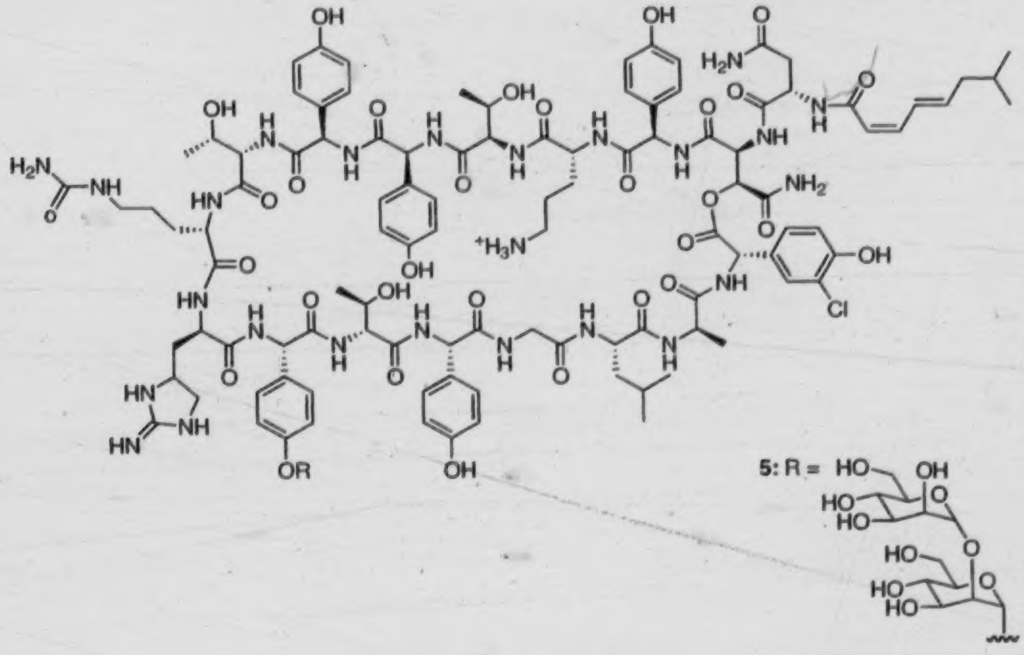


$$\begin{array}{l}CH_3\text{-}\underset{CH_3}{CH}\text{-}(CH_2)_{10}\text{-}CH\text{-}CH_2\text{-}CO\text{-allo-D-Thr-D-Ala-HyPro-L-Arg}\\ \qquad\qquad\qquad\qquad | \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad |\\ \qquad\qquad\qquad\quad O \text{———— HyAsp}^1\text{-HyPro-D-Ser-HyAsp}^2\end{array}$$

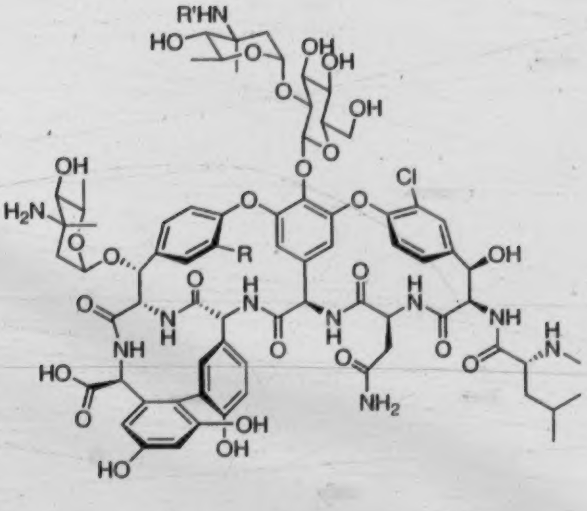
4 HyAsp¹ = L-*threo*-β-hydroxyaspartic acid
HyAsp² = D-*threo*-β-hydroxyaspartic acid
HyPro = L-*trans*-3-hydroxyproline

Many series of novel glycopeptide analogs with activity on vancomycin resistant bacteria have also been identified. Ramoplanin (**5**) (Figure 5)[101-103] inhibits GTase primarily by lipid II sequestration. Even though they all share a common fold and function, the cyclic glycodepsipeptide **5** differs from other GTase inhibitors such as moenomycin and mersacidin in that it not only kills Gram-positive bacteria by inhibition of GTase activity, but also by generation of a metabolic block at the level of the MurG reaction forcing an increase of cellular pools of lipid I.[104,105] LY333328 (**6**) and LY191145 (**7**)[106-108] are glycopeptide antibiotics based on the parent structures of eremomycin (**8**) and chloroeremomycin (**9**) (Figure 5). Compound **6** inactivates a transglycosylation unit by binding to D-Ala-D-Ala present in lipid II. It is highly active against methicillin-resistant *S. aureus* and vancomycin-resistant enterococci (VRE), and is now in clinical trials.[109,110]

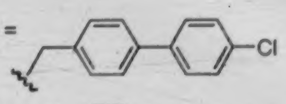
Figure 5. Structure of ramoplanin and analogs of eremomycin and chloroeremomycin



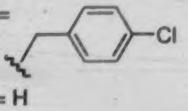
5: R =



6: R = Cl; R' =



7: R = Cl; R' =

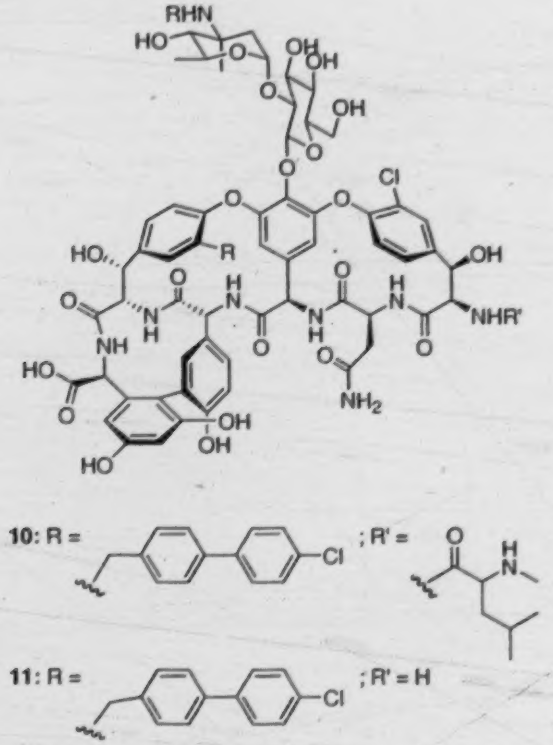


8: R = H; R' = H

9: R = Cl; R' = H

A series of vancomycin analogs has also been reported as inhibitors of GTase (Figure 6).[111] Chlorobiphenyl-vancomycin (**10**) displays two modes of action, *i.e.* binding to D-Ala-D-Ala and inhibition of transglycosylation in the absence of dipeptide binding. Chlorobiphenyl-desleucyl-vancomycin (**11**) does not require dipeptide binding to prevent the transglycosylation process from happening.[112,113]

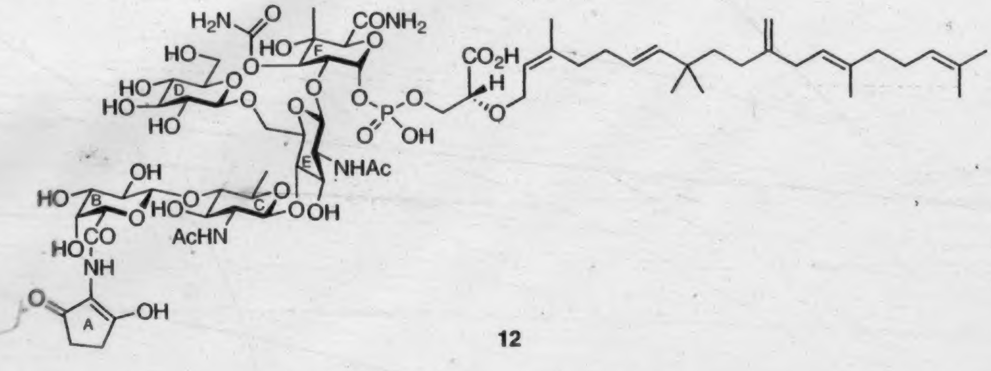
Figure 6. Structure of vancomycin analogs



2.2.2. Moenomycin Analogs

Of the moenomycin-type compounds, moenomycin A (**12**) (Figure 7),[114] the main component of the trade product Flavomycin®, is one of the most potent GTase inhibitors known. Its biosynthesis has been studied[115-117] and its three-dimensional structure established.[118,119] The mechanism for its mode of action is believed to proceed by anchoring to the cytoplasmic membrane *via* its lipid chain, essential for inhibition of GTase activity,[120-123] followed by highly selective recognition of its oligosaccharide moiety at a substrate binding site of the enzyme.[79,124] Extensive structure-activity relationship studies of moenomycin analogs have been done.[120,125-133] Systematic degradation studies have revealed that moenomycin analogs with at least three carbohydrate units (C, E, and F) are active *in vivo* against Gram-positive bacteria. Moenomycin disaccharide derivatives (lacking units A, B, C and D) are also active GTase inhibitors,[134] though they lack antibacterial activity.[135] Novel disaccharide antimicrobial agents based on moenomycin have been discovered using combinatorial chemistry.[136]

Figure 7. Structure of moenomycin A (**12**)



12

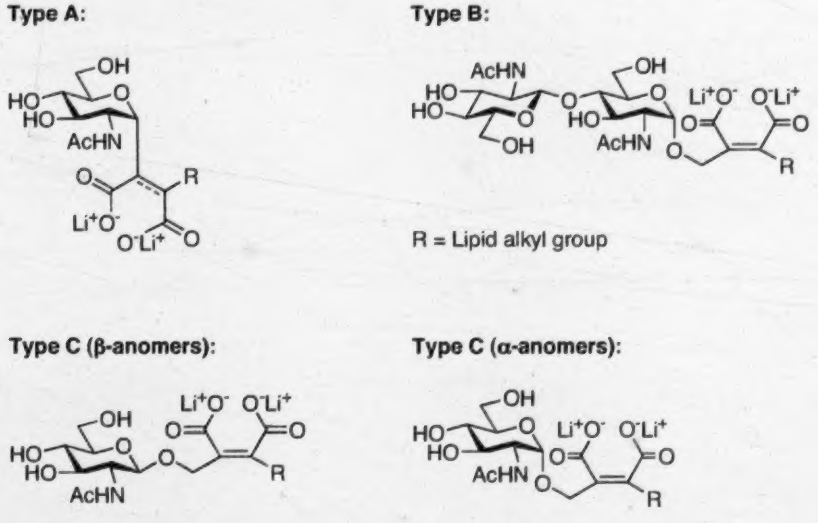
2.3. Screening Systems for Identifying Inhibitors of Bacterial GTase

The development of new screening systems necessitates the search for better substrates for bacterial GTase.[137] The GTase is one of the most difficult enzymes to assay. In the past five years, various assays have been reported for detecting murein GTase inhibitors. An in situ assay using cell wall membrane material isolated from *E. coli*, exogenously supplied UDP-MurNAc-pentapeptide, and radiolabeled UDP-GlcNAc specifically identifies GTase inhibitors.[138] An assay based on competition experiments that use surface plasmon resonance can also be used to investigate selective binding of moenomycin analogs and other GTase inhibitors.[139] A simple competition assay based on the specific binding of moenomycin to PBPs employing Affi-Gel beads and a simple filtration procedure is also reported.[140] Screening systems utilizing *E. faecalis* strain A256 and *E. faecalis* strain MDD212 allow detection of inhibitors of cell wall transglycosylation in *Enterococcus* by rescue of the cells and induction of β-galactosidase in the presence of inhibitors, respectively.[141] Two very recently developed thin layer chromatography (TLC) screening systems can also be used for the detection of GTase inhibitors among samples of crude preparations.[142]

3. Project Goals: Studies, Design and Synthesis of Potential GTase Inhibitors

Two approaches are undertaken in this thesis to study potential inhibitors of the peptidoglycan biosynthesis. The objective of the first part of this work is to investigate new types of mono- and disaccharide derivatives for GTase inhibition. Three types of derivatives of the peptidoglycan monomer unit possessing a dicarboxylate moiety as a mimic of the diphosphate of lipid II have been designed as potential inhibitors of GTase (Figure 8). Targets **A** contain a non-cleavable α-*C*-glycoside, a stable surrogate for the α-*O*-glycoside, and could serve to probe the distance requirements between the sugar and the diacid moiety. Type **B** compounds resemble the natural substrate of GTase, lipid II, and are targeted to examine the importance of the second sugar moiety. Targets **C** may provide insight into the importance of the α-linkage at the anomeric center. All three targets, even though lacking the physiologically active peptide chain required for biological activity in mammals, contain some characteristics expected for recognition by GTase including a lipid tail and a terminal GlcNAc unit. If incorporated into peptidoglycan, these compounds could potentially "end-cap" the growing polysaccharide chain.

Figure 8. Synthetic target molecules: derivatives of lipid II



Type A:

Type B:

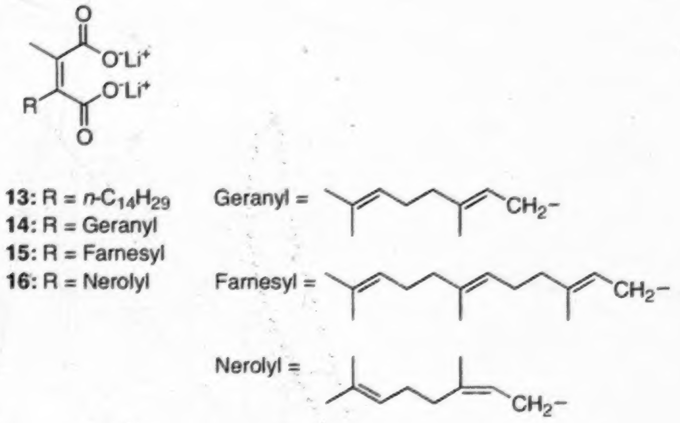
R = Lipid alkyl group

Type C (β-anomers):

Type C (α-anomers):

Chaetomellic acid A analogs **13-16** were also of interest (Figure 9). It is known that chaetomellic acid A is a good inhibitor of Ras protein farnesyltransferases. The designed targets resemble the undecaprenyl diphosphate moiety of lipid II. In addition to their ability to serve as glycosyl acceptors for the synthesis of type **A** targets, compounds **13-16** could potentially inhibit rubber transferase, a *cis*-prenyltransferase. Even though the study of protein prenyltransferases is not the main subject of this thesis, as the targeted compounds have potential inhibitory properties against such enzymes, the design and results of both synthetic studies and biological testing of these compounds are described in the next section.

Figure 9. Structure of targets **13-16**



13: R = n-$C_{14}H_{29}$
14: R = Geranyl
15: R = Farnesyl
16: R = Nerolyl

The goal of the second part of this manuscript is to study various bacteriocins of lactic acid bacteria (*e.g.* piscicolin 126, carnobacteriocins B1 and BM1, and brochocin C) that may inhibit GTase by binding to lipid II.

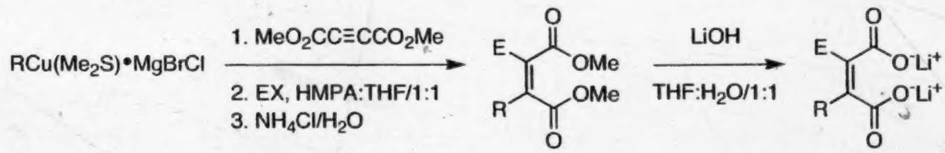
RESULTS AND DISCUSSION

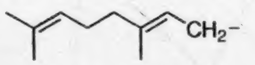
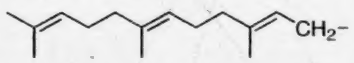
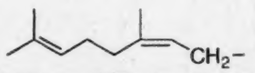
1. Chaetomellic Acid A Analogs as Inhibitors of Rubber Transferase

1.1. Synthesis of Chaetomellic Acid A Derivatives 13-22

Chaetomellic acid A (**19**), isolated from *Chaetomella acutiseta*,[143] is an inhibitor of Ras protein farnesyltransferases (PFTases)[144] and competes for the farnesyl diphosphate (FPP) binding site of PFTases with a IC_{50} of 55 nM.[145] Compounds **13-22** were prepared according to the method previously developed in our group by Ratemi *et al.* (Table 2).[146] Michael addition of various organocopper reagents to DMAD in the presence of HMPA, followed by capture of the resulting enolates with a variety of electrophiles affords chaetomellic acid analogs **17-22** as methyl esters in 73-87% yield. Ester hydrolysis with lithium hydroxide furnishes derivatives **13-16** in 90-99% yield.

Table 2. Preparation of chaetomellic acid A analogs by conjugate addition to DMAD



R	EX	E	Product	Yield (%)[a]	Hydrolysis product	Yield (%)
n-C_8H_{17}	---	H	**17**	87	---	---
n-$C_{14}H_{29}$	---	H	**18**	85	---	---
n-$C_{14}H_{29}$	MeI	Me	**19**	76	**13**	99
Me	Geranyl-Br		**20**	84	**14**	90
Me	Farnesyl-Br		**21**	82	**15**	99
Me	Nerolyl-Br[b]		**22**	73	**16**	99

[a] Isolated yields. [b] Freshly prepared from nerol.

1.2. Rubber Transferase Inhibition Studies with Chaetomellic Acid A Analogs 13-16

Upon completion of their syntheses compounds **13-16** were tested as inhibitors for rubber transferase, a *cis*-prenyltransferase that catalyzes the biosynthesis of natural rubber.[147] Inhibition studies against rubber transferases from *Hevea brasiliensis* and *Parthenium argentatum* were performed by Christopher J. D. Mau (USDA, Agricultural Research Service, Western Regional Research Center, Albany, CA), using a multiwell filtration system.[148] Dimethylallyl diphosphate (DMAPP) and FPP were used as competing initiators.

The results[149] of enzyme inhibition tests against rubber transferase from *H. brasiliensis* reveal that chaetomellic acid A (**13**) is inhibitory when present at 20 μM in up to 20- or 200-fold molar excess of DMAPP or FPP, respectively. Compound **15** inhibits activity by 25% when included in the assay at 180 μM in 1800-fold molar excess of FPP. When tested against rubber transferase from *P. argentatum* compounds **13** and **15** reduce activity by 48% (when added at 180 μM in a 180-fold molar excess) and by 27% (when present at 180 μM in a 18,000-fold molar excess), respectively. Compounds **14** and **16** fail to exhibit any inhibition against both species of rubber transferase when present at 180 μM in over a million-fold molar excess of initiator.

The results of kinetic experiments, summarized in Table 3, indicate that compounds **13** and **15** behave as competitive inhibitors of the *P. argentatum* rubber transferase. In contrast, although chaetomellic acid A (**13**) competitively inhibits the *H. brasiliensis* rubber transferase, compound **15** acts in a non-competitive manner.

Table 3. Kinetic constants for the interaction between compounds **13** and **15** with *H. brasiliensis* and *P. argentatum* rubber transferases

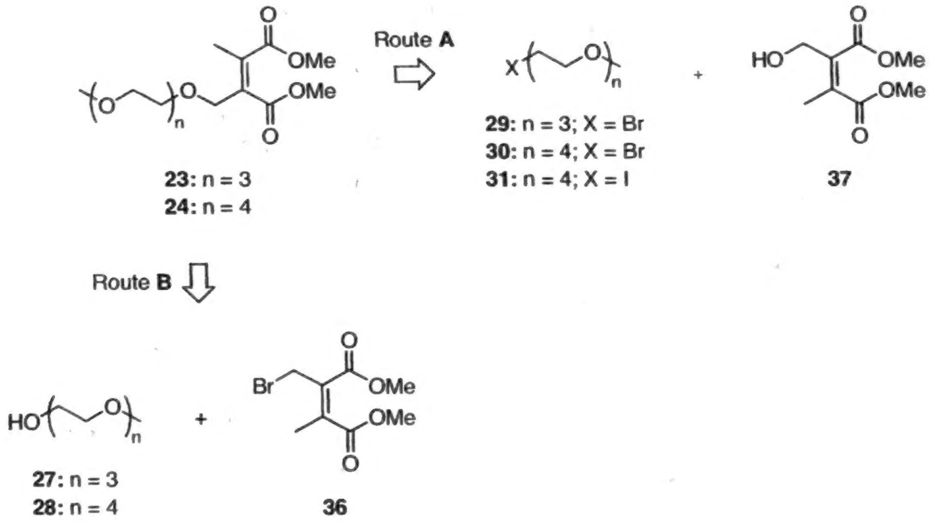
Compound	***H. brasiliensis***		***P. argentatum***	
	K_i (μM)	***Type of Inhibitor***	***K_i (μM)***	***Type of Inhibitor***
13	42	competitive	8.8	competitive
15	140	non-competitive	25	competitive

In summary, the results show that the length of the hydrophobic carbon tail plays an important role in determining efficacy of inhibition, which supports the proposed model for the rubber transferase active site.[147,150] The results also indicate that although rubber transferases from evolutionarily divergent species share many commonalities differences exist in the geometry of their catalytic sites.

1.3. Attempts at the Synthesis of Chaetomellic Acid A Analogs 23 and 24

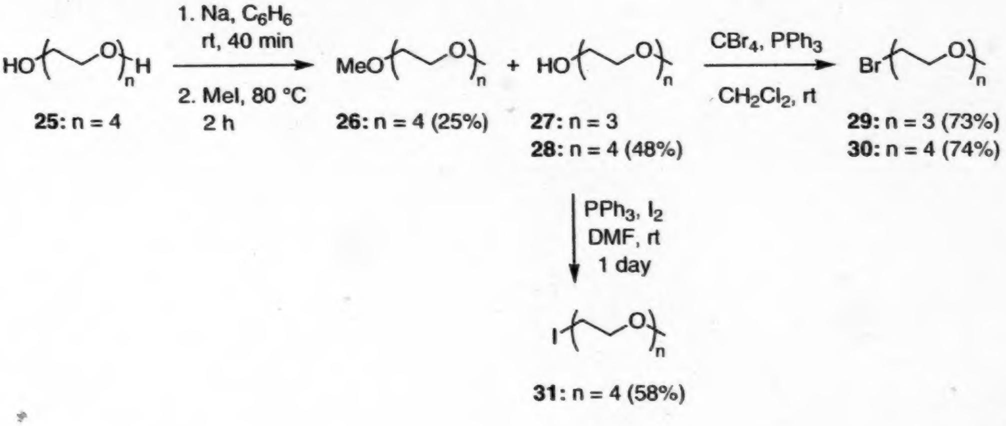
Chaetomellic acid A derivatives **23** and **24** bearing a polyether chain in place of the hydrophobic carbon tail are likely to have better physical properties, such as water solubility, than analogs **13-22**. Retrosynthetic analysis of compounds **23** and **24** indicates that two convergent strategies can be used for their preparation (Scheme 1). Route **A** affords target molecules **23** and **24** from key intermediates **29-31** and **37**. The halogenated polyethers **29-31** and alcohol **37** can be readily prepared from the building blocks **27**, **28** and **36** of route **B**. Both routes outlined in Scheme 1 were attempted in order to synthesize the desired molecules.

Scheme 1. Retrosynthetic analysis for the preparation of analogs **23** and **24**



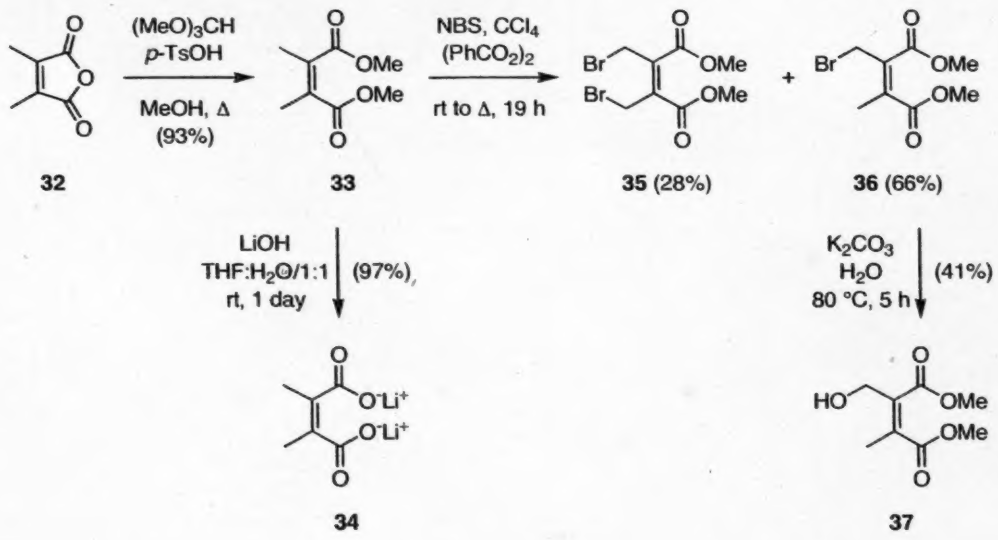
The synthesis of key intermediates **28-31** required for both routes is outlined in Scheme 2. Following a procedure described by Ercolani *et al.*,[151] tetraethylene glycol (**25**) is heated in benzene with sodium and methyl iodide to afford the desired monomethyl ether (**28**) as the major product (48%) and the dimethylated side-product **26**. Several bromination methods have been reported in the literature and were applied to the synthesis of bromide **29**. When treated with phosphorus tribromide in diethyl ether,[152,153] alcohol **28** produces the desired bromide **30** in 12% yield. Upon treatment with triphenylphosphine and bromine in acetonitrile,[154] alcohol **28** gives compound **30** in 21% yield. The reaction conditions described by Hayashi *et al.* were found to give the best results.[155] Alcohols **27** and **28** react with triphenylphosphine and carbon tetrabromide to produce the corresponding bromides **29** and **30** in reasonable yield (73-74%). Formation of iodide **31** is realized by treatment of alcohol **28** with iodine and triphenylphosphine in DMF in 58% yield.

Scheme 2. Synthesis of key intermediates **28-31**


HO(CH2CH2O)nH
1. Na, C6H6
rt, 40 min
2. MeI, 80 °C
2 h
MeO
+
HO
CBr4, PPh3
CH2Cl2, rt
Br
PPh3, I2
DMF, rt
1 day
I
25: n = 4
26: n = 4 (25%)
27: n = 3
28: n = 4 (48%)
29: n = 3 (73%)
30: n = 4 (74%)
31: n = 4 (58%)

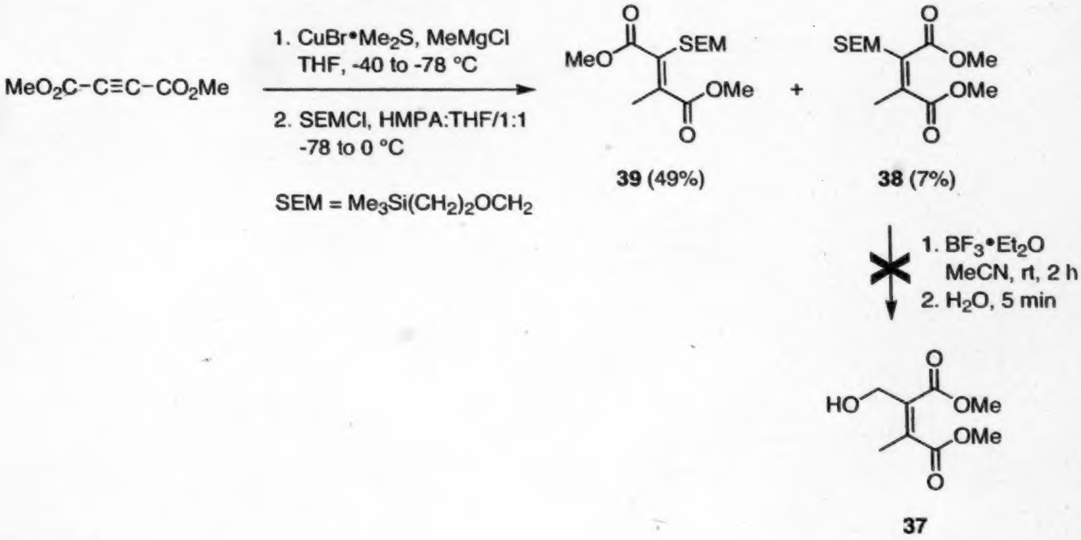
The preparation of key intermediates **36** and **37** is outlined in Scheme 3. Diester **33** was synthesized from commercially available dimethylmaleic anhydride (**32**) by modification of the literature procedure described by Müller and Rodriguez.[156] Anhydride **32** reacts under reflux with trimethyl orthoformate and *p*-toluenesulfonic acid to give the expected diester **33**, which upon hydrolysis with lithium hydroxide produces the corresponding lithium salt **34** in 97% yield. Treatment of diester **33** with *N*-bromosuccinimide and dibenzoyl peroxide in carbon tetrachloride provides the desired bromide **36** (66%) as a major product in conjunction with the known dibromide **35** (28%) as a side-product. Conversion of bromide **36** to the desired volatile alcohol **37** (41%) is finally achieved using potassium carbonate in water.

Scheme 3. Synthesis of key intermediates **36** and **37**


(MeO)3CH
p-TsOH
MeOH, Δ
(93%)
NBS, CCl4
(PhCO2)2
rt to Δ, 19 h
LiOH
THF:H2O/1:1
rt, 1 day
(97%)
K2CO3
H2O
80 °C, 5 h
(41%)
OMe
O⁻Li⁺
Br
HO
32
33
35 (28%)
36 (66%)
34
37

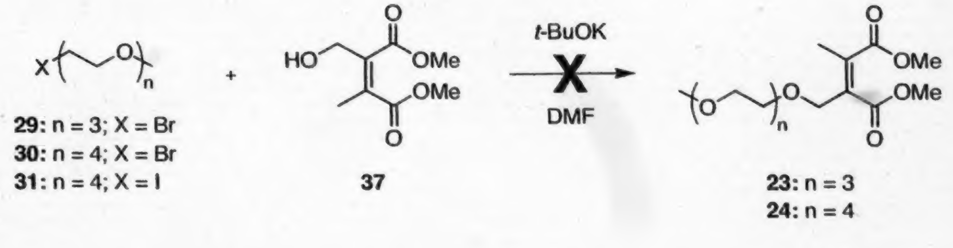
In an effort to improve upon overall yield and acquire alcohol **37** in a more efficient manner, a modification of the conjugate addition-enolate trapping methodology described by Ratemi *et al.*[146] was explored as an alternative synthetic path (Scheme 4). Reaction of CuBr•Me_2S, MeMgCl and DMAD in the presence of HMPA, followed by alkylation of the conjugate adduct with a trimethylsilylethoxymethyl chloride (SEMCl) gives a separable mixture of *Z* and *E* protected compounds **38** (7%) and **39** (49%) in which the desired *Z* isomer **38** is the minor component, as confirmed by NOE experiments. As the yield in this step is unsatisfactory and the attempt at removing the trimethylsilylethoxymethyl group using BF_3•Et_2O fails to give the desired alcohol **37**, this method was abandoned.

Scheme 4. Attempt at the synthesis of **37**


MeO$_2$C−C≡C−CO$_2$Me
1. CuBr•Me$_2$S, MeMgCl THF, -40 to -78 °C
2. SEMCl, HMPA:THF/1:1 -78 to 0 °C
SEM = Me$_3$Si(CH$_2$)$_2$OCH$_2$
MeO
SEM
OMe
O
+
OMe
39 (49%)
38 (7%)
1. BF$_3$•Et$_2$O MeCN, rt, 2 h
2. H$_2$O, 5 min
HO
37

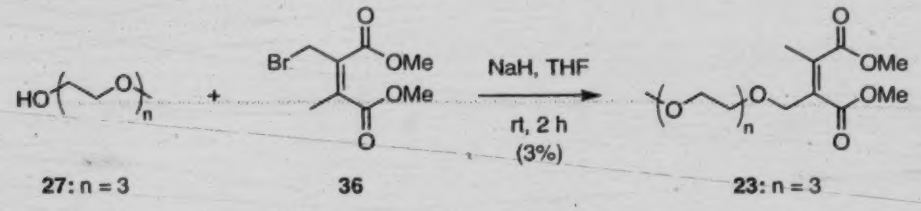
The next step in these syntheses involves coupling of the various building blocks. Literature precedents for route **A**[157] led us to attempt attack of the halogenated polyethers with the anion of alcohol **37** (Scheme 5). Unfortunately, reaction of compounds **29-31** with alcohol **37** in the presence of potassium *tert*-butoxide in DMF fails to produce the desired coupled products **23** and **24**.

Scheme 5. Attempted coupling of **29-31** with alcohol **37**


X
O
n
29: n = 3; X = Br
30: n = 4; X = Br
31: n = 4; X = I
+
HO
OMe
OMe
37
t-BuOK
DMF
23: n = 3
24: n = 4

The above results indicate that nucleophilic attack of a halogenated polyether as suggested in route **A** is not a viable approach to obtain chaetomellic acid A derivatives **23** and **24**. In the last five years a large number of examples have been reported in the literature for the displacement of a bromide by the anion of a tri- or tetraethylene glycol monomethyl ether.[158-163] After many attempts at coupling alcohol **27** and **28** to bromide **36** using various solvents and temperature conditions, chaetomellic acid A analog **23** could be detected by ^{1}H NMR and mass analyses (Scheme 6). Treatment of alcohol **27** with NaH at room temperature generates the anion that is reacted with bromide **36** to give the desired coupled product **23** in very low yield (3%). Unfortunately, complete purification by HPLC could not be achieved.

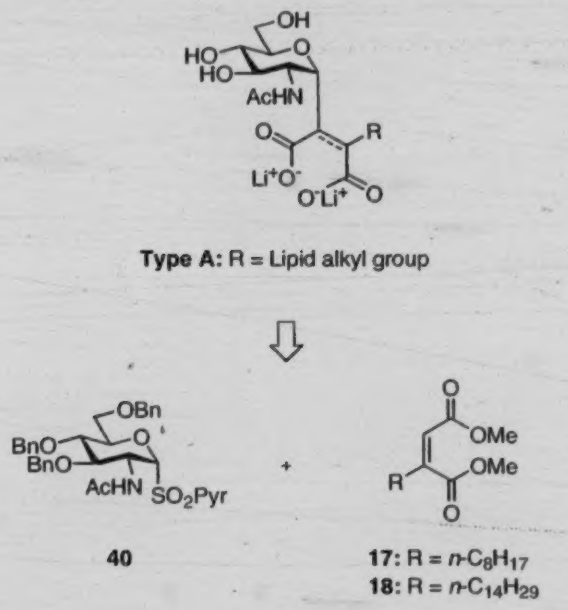
Scheme 6. Synthesis of **23** from coupling of alcohol **27** with bromide **36**



2. Synthetic Studies Toward Type A Targets: α-*C*-Glycosides

Over the past two decades, carbon-linked glycosyl compounds have become important biological tools for the study of carbohydrate-protein interactions. Their chemical stability and their favorable conformational properties have stimulated the development of a wide range of synthetic methods,[164-169] including syntheses based on 2-amino and 2-acetamido sugars mediated by free radicals.[170-172] The retrosynthesis of type A targets (Scheme 7) is based on the work of Urban *et al.*[173] on stereocontrolled syntheses of α-*C*-galactosamine derivatives *via* reductive samariation. From a retrosynthetic perspective, type **A** compounds may be formed *via* glycosylation of a suitably protected glycosyl donor carrying a 2-pyridyl sulfone at the anomeric center **40** with maleate derivatives **17** and **18** already synthesized (Table 2).

Scheme 7. Retrosynthetic analysis for the preparation of type **A** targets



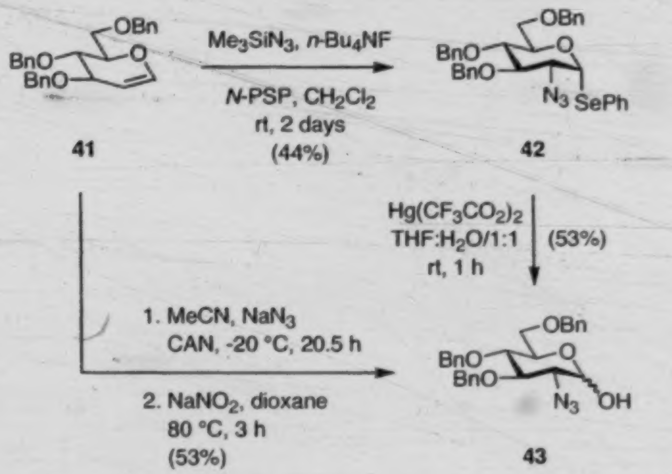
Type A: R = Lipid alkyl group

40

17: R = n-C_8H_{17}
18: R = n-$C_{14}H_{29}$

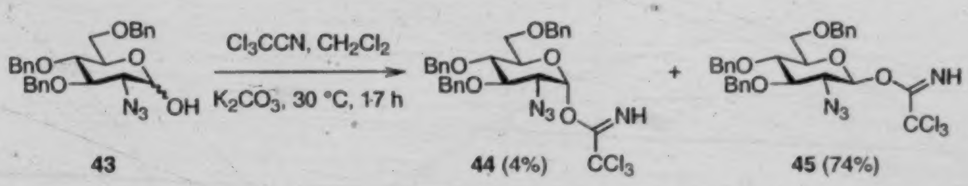
The synthesis of glycosyl donor **40** begins with the preparation of reducing sugar **43**, which can be obtained from 3,4,6-tri-*O*-benzyl-*D*-glucal (**41**) *via* two different approaches (Scheme 8). Based on a procedure described by Kinzy and Schmidt,[174] compound **41** is directly converted to alcohol **43** by treatment with sodium azide, ceric ammonium nitrate and sodium nitrite, in 53% yield. The poor quality, low purity, of the product thus obtained led us to examine another way for synthesizing reducing sugar **43**. The methodology described by Czernecki and Ayadi was applied.[175] Glucal **41** reacts with tetrabutylammonium fluoride, azidotrimethylsilane and *N*-phenylselenophthalimide in dichloromethane to give the expected selenide **42**, which is subsequently treated with mercury trifluoroacetate to afford the desired alcohol **43**, in 23% overall yield. The product of this reaction is of better quality and was used to continue on the synthesis of glycosyl donor **40**.

Scheme 8. Conversion of 3,4,6-tri-*O*-benzyl-*D*-glucal (**41**) to alcohol **43**


BnO
BnO
OBn
O
41
Me3SiN3, n-Bu4NF
N-PSP, CH2Cl2
rt, 2 days
(44%)
BnO
BnO
OBn
O
N3 SePh
42
Hg(CF3CO2)2
THF:H2O/1:1
rt, 1 h
(53%)
1. MeCN, NaN3
CAN, -20 °C, 20.5 h
2. NaNO2, dioxane
80 °C, 3 h
(53%)
BnO
BnO
OBn
O
N3 OH
43

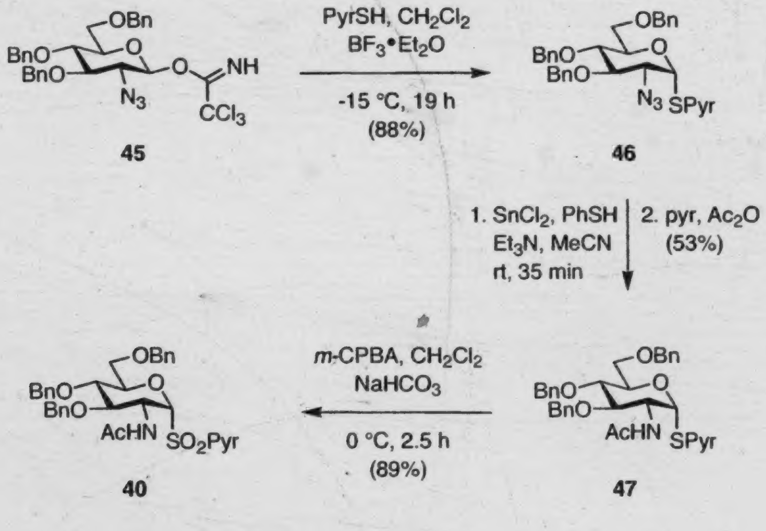
Subsequent imidation of **43** with trichloroacetonitrile and potassium carbonate in dichloromethane produces the desired β-trichloroacetimidate **45** and the α-anomer **44** in 74% and 4% yield, respectively (Scheme 9).

Scheme 9. Formation of α- and β-trichloroacetimidate **44** and **45** from alcohol **43**


BnO
BnO
OBn
O
N3 OH
43
Cl3CCN, CH2Cl2
K2CO3, 30 °C, 17 h
BnO
BnO
OBn
O
N3 O
NH
CCl3
44 (4%)
+
BnO
BnO
OBn
O
O
NH
N3
CCl3
45 (74%)

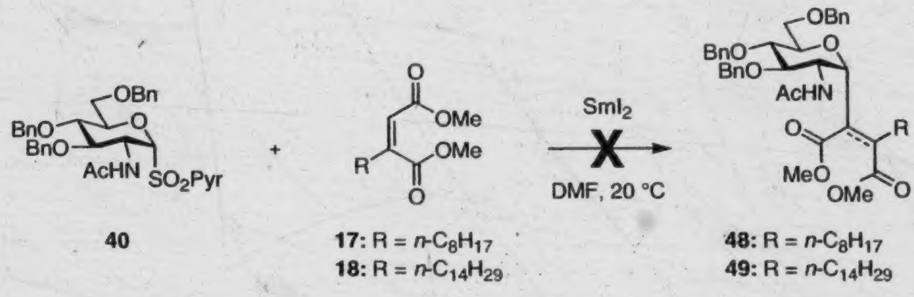
The synthesis was continued with β-trichloroacetimidate **45** (Scheme 10). The α-oriented 2-pyridyl sulfide **46** (88%) is obtained by treatment of β-trichloroacetimidate **45** with 2-mercaptopyridine and BF_3•Et_2O. Several methods to perform the azide reduction reaction have been reported in the literature, within which, reaction conditions including nickel chloride and sodium borohydride,[176] as well as samarium iodide,[177,178] have found broad application. However, the chosen procedure involves the use of tin chloride as a reducing agent.[179] Thus, reduction of azide **46** using tin chloride, thiophenol and triethylamine, followed by treatment of the resulting amine with pyridine and acetic anhydride overnight affords acetamide **47** in 53% yield. Oxidation of the sulfide moiety of **47** with *m*-chloroperbenzoic acid in the presence of sodium bicarbonate produces the desired sulfone **40** in 89% yield.

Scheme 10. Synthesis of glycosyl donor **40** from β-trichloroacetimidate **45**


OBn
BnO
BnO
O
O
NH
N3
CCl3
45
PyrSH, CH2Cl2
BF3•Et2O
-15 °C, 19 h
(88%)
OBn
BnO
BnO
O
N3
SPyr
46
1. SnCl2, PhSH
Et3N, MeCN
rt, 35 min
2. pyr, Ac2O
(53%)
OBn
BnO
BnO
O
AcHN
SO2Pyr
40
m-CPBA, CH2Cl2
NaHCO3
0 °C, 2.5 h
(89%)
OBn
BnO
BnO
O
AcHN
SPyr
47

With the successful preparation of sulfone **40**, the next step consists of coupling with compound **17** and **18** (Scheme 11). Unfortunately, attempted synthesis of α-*C*-glucosamines **48** and **49** by glycosylation of sulfone **40** with aglycons **17** or **18** mediated by samarium iodide fails to give any coupled product. Compounds **17** and **18** were recovered unchanged.

Scheme 11. Attempted coupling of sulfone **40** with aglycons **17** and **18**



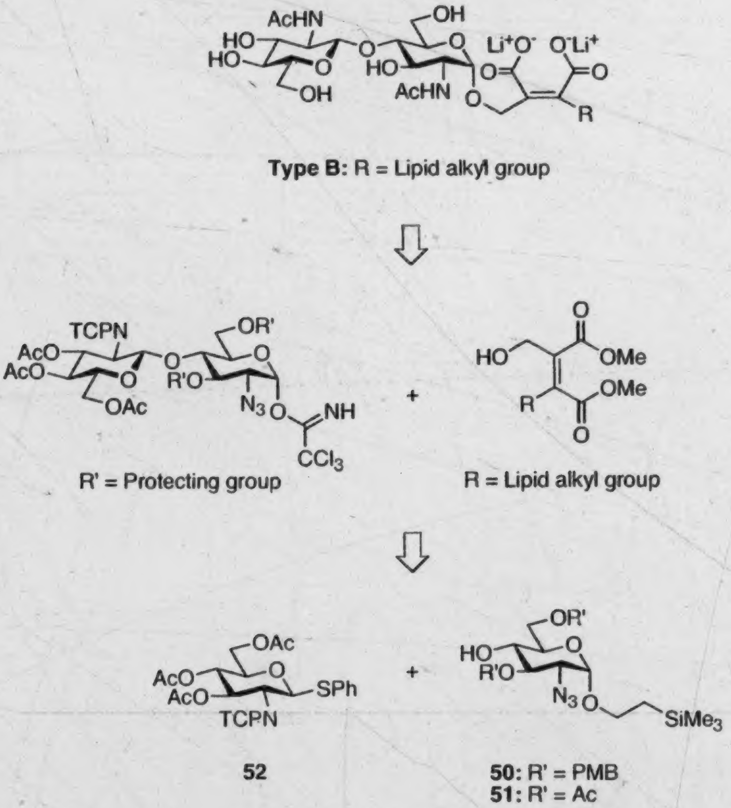
Future work on the synthesis of type **A** target could be based on the use of anomeric selenides and radical generation by tributyl tin hydride as recently described by SanMartin *et al.* for the preparation of *C*-glycoside analogs of *O*-benzyl α-*D*-GalNAc.[172]

3. Synthetic Studies Toward Type B Targets: Disaccharides

Most studies found in the literature on potential inhibitors of the bacterial cell wall GTases are based on the synthesis of moenomycin A analogs. However, the natural GTase substrate, lipid II, also provides a structural motif for inhibitor design. In recent years, our group and others have focused our attention on the design of analogs of the lipid intermediates of peptidoglycan biosynthesis. The preparation of *N*-acetylglucosamine thiazolines as lipid II hybrids has been reported.[180] A paper by Silva and co-workers on the synthesis and biological evaluation of analogs of lipid I indicates that the anomeric lipid phosphate plays a key role in substrate recognition and processing.[65] Our type **B** targets closely resemble the GTase substrate with a lipid alkyl chain and a terminal *D*-glucosamine unit expected for recognition.

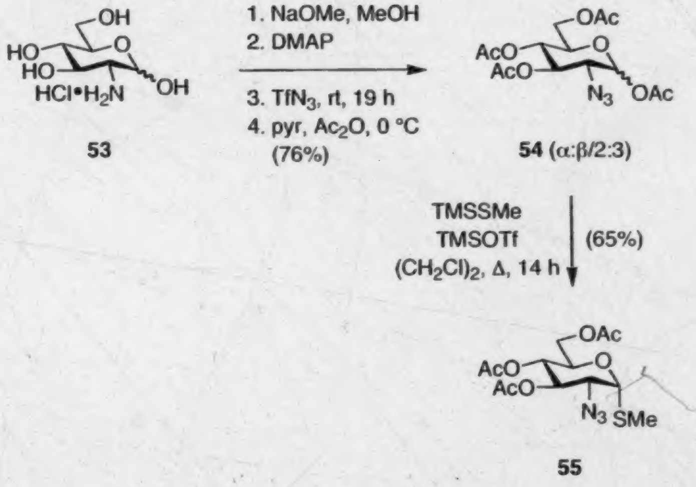
The synthetic strategy for the construction of inhibitors of type **B** is based on the retrosynthetic analysis outlined in Scheme 12. The target molecules may be derived from maleate derivatives already carrying the lipid alkyl chain, and a disaccharide imidate accessible *via* glycosylation of a suitably protected *O*-glucoside (**50** or **51**) with a *D*-glucosaminyl donor **52**.

Scheme 12. Retrosynthetic analysis for the preparation of type **B** targets


AcHN
HO
HO
OH
OH
O
HO
AcHN
Li+O-
O-Li+
O
R
Type B: R = Lipid alkyl group
TCPN
AcO
AcO
OAc
OR'
R'O
N3
NH
CCl3
R' = Protecting group
+
HO
OMe
OMe
R
R = Lipid alkyl group
OAc
AcO
AcO
SPh
TCPN
52
HO
R'O
N3
SiMe3
50: R' = PMB
51: R' = Ac

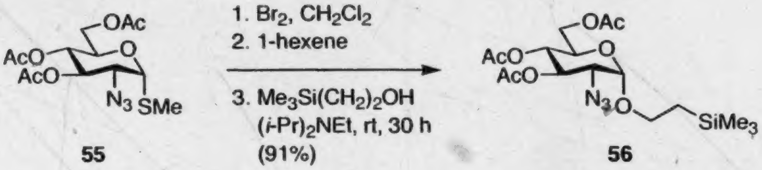
The synthesis of the disaccharide intermediate begins with the preparation of the glycosyl acceptor **50**, which can be obtained from thioglucoside **55**. The transformation of *D*-glucosamine hydrochloride (**53**) to glycosyl donor **55** is outlined in Scheme 13. Treatment of **53** with sodium methoxide, 4-(dimethylamino)pyridine and freshly prepared triflic azide gives 2-azido-2-deoxy-*D*-glucose, which is acetylated by pyridine and acetic anhydride to provide **54** as a mixture of anomers (α:β/2:3) in 76% yield. Azide **54** is heated under reflux in the presence of (methylthio)trimethylsilane and trimethylsilyl triflate,[181] to give thioglucoside **55** in 65% yield.

Scheme 13. Synthesis of thioglucoside **55** from *D*-glucosamine hydrochloride (**53**)


OH
HO
O
HO
HCl•H2N
OH
53
1. NaOMe, MeOH
2. DMAP
3. TfN3, rt, 19 h
4. pyr, Ac2O, 0 °C
(76%)
OAc
AcO
O
AcO
N3
OAc
54 (α:β/2:3)
TMSSMe
TMSOTf
(CH2Cl)2, Δ, 14 h
(65%)
OAc
AcO
O
AcO
N3
SMe
55

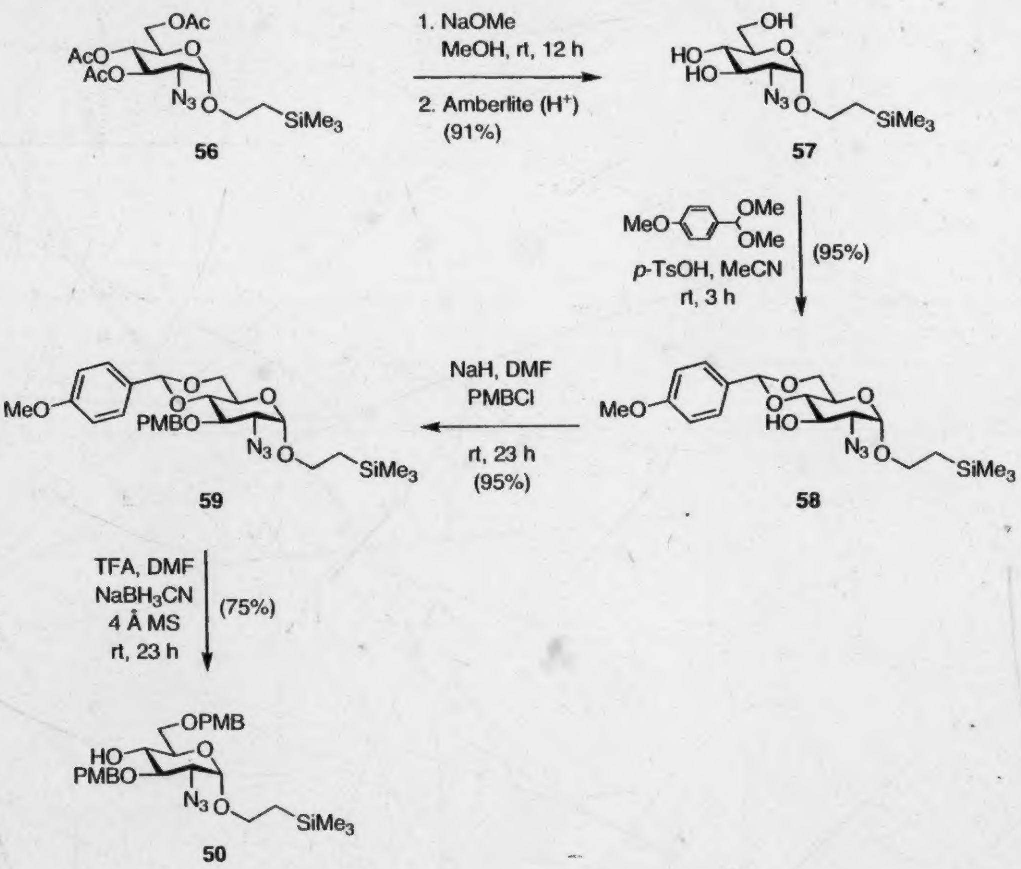
One of the key structural features of the targeted glycosyl acceptor **50** is its protecting group at the anomeric center. 2-(Trimethylsilyl)ethyl was chosen to protect the anomeric position of thioglucoside **55**, as it is stable to most reaction conditions and easily removed by the use of $BF_3 \bullet Et_2O$ (Scheme 14). Activation of **55** with bromine in dichloromethane followed by addition of (trimethylsilyl)ethanol gives the desired protected product **56** in 91% yield.

Scheme 14. Protection of thioglucoside **55** with (trimethylsilyl)ethanol



The selective protection of positions 3 and 6 of the desired glycosyl acceptor **50** can be realized from sugar **56** by the series of transformations depicted in Scheme 15. Compound **56** is first deacetylated by treatment with sodium methoxide to give **57** in 91% yield. Selective protection of OH-4 and OH-6 of **57** using anisaldehyde dimethylacetal and *p*-toluenesulfonic acid produces *p*-methoxybenzylidene **58** in 95% yield. Subsequent protection of the free hydroxyl group at C-3 of **58** using *p*-methoxybenzyl chloride and sodium hydride in *N,N*-dimethylformamide gives the fully protected monosaccharide **59** in 95% yield. Finally, the required free OH-4 is obtained through the regioselective opening of the acetal ring of **59**. Treatment of **59** with sodium cyanoborohydride and trifluoroacetic acid in the presence of 4 Å molecular sieves gives the desired glycosyl acceptor **50** in 75% yield.

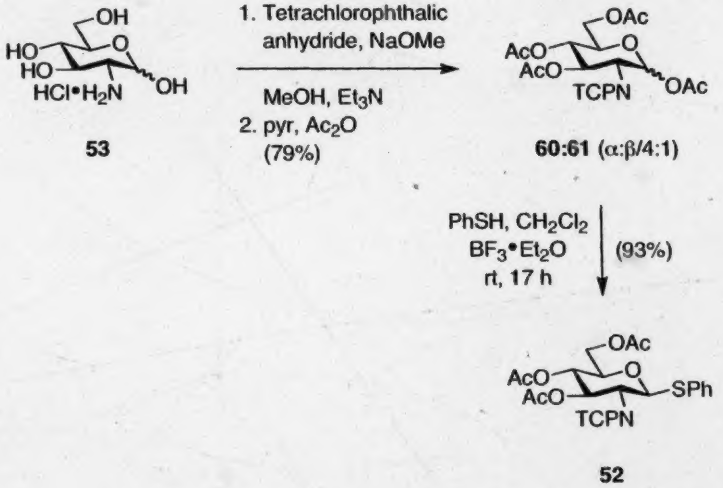
Scheme 15. Preparation of glycosyl acceptor **50** from **56**


OAc
AcO
AcO
O
N3
O
SiMe3
56
1. NaOMe
MeOH, rt, 12 h
2. Amberlite (H+)
(91%)
OH
HO
HO
O
N3
O
SiMe3
57
MeO
OMe
OMe
p-TsOH, MeCN
rt, 3 h
(95%)
MeO
O
O
O
HO
N3
O
SiMe3
58
NaH, DMF
PMBCl
rt, 23 h
(95%)
MeO
O
O
O
PMBO
N3
O
SiMe3
59
TFA, DMF
NaBH3CN
4 Å MS
rt, 23 h
(75%)
OPMB
HO
PMBO
O
N3
O
SiMe3
50

With the successful elaboration of the glycosyl acceptor **50** complete, we focused our attention on the preparation of glycosyl donor **52**. Several synthetic methodologies have been developed to incorporate the 2-acetamino-2-deoxy-β-*D*-glycopyranoside moiety in β-1,4-linked disaccharides of type **B**. The formation of glycosidic linkages for the preparation of various disaccharides has been reviewed.[182] Among the glycosyl donors reported for the synthesis of β-1,4-linked disaccharides, the known thioglucoside **52** containing an easily removed *N*-tetrachlorophthaloyl functionality was chosen and prepared according to the method described by Castro-Palomino and Schmidt (Scheme

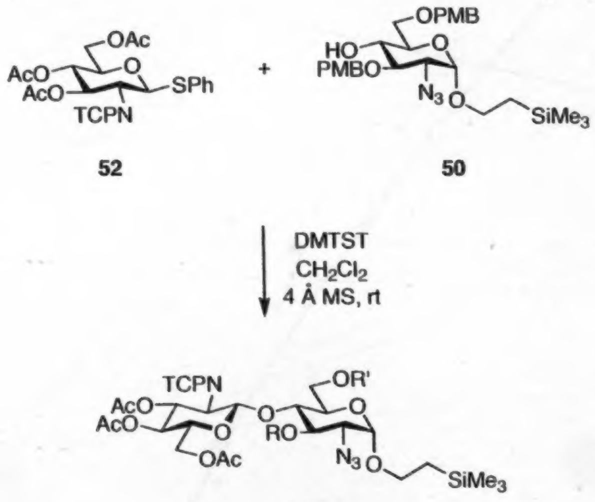
16).[183] Treatment of *D*-glucosamine hydrochloride (**53**) with *N*-tetrachlorophthalic anhydride, sodium methoxide and triethylamine followed by reaction with pyridine and acetic anhydride gives the acetylated tetrachlorophthalimide as a mixture of anomers (α:β/4:1) in 79% yield. Reaction of the mixture of anomers **60** and **61** with thiophenol and BF_3•Et_2O results in the formation the desired glycosyl donor **52** in 93% yield.

Scheme 16. Synthesis of glycosyl donor **52** from *D*-glucosamine hydrochloride (**53**)


OH
HO
O
HO
HCl•H₂N
OH
53
1. Tetrachlorophthalic anhydride, NaOMe
MeOH, Et₃N
2. pyr, Ac₂O
(79%)
OAc
AcO
O
AcO
TCPN
OAc
60:61 (α:β/4:1)
PhSH, CH₂Cl₂
BF₃•Et₂O
rt, 17 h
(93%)
OAc
AcO
O
AcO
TCPN
SPh
52

Initial attempts at the synthesis of the desired disaccharide **62** were done with donor **52** and acceptor **50** with the intention of maintaining the hydroxyl groups at position 3 and 6 of the glycosyl acceptor functionalized as the *p*-methoxybenzyl ethers. Activation of thioglycosides can be accomplished with a variety of promoters including NIS with silver trifluoromethanesulfonate,[184] methyl trifluoromethanesulfonate, and dimethyl(methylthio)sulfonium trifluoromethanesulfonate (DMTST).[185] DMTST, an unstable white solid that must be kept at -20 °C, is prepared in 2 days from dimethyl disulfide and methyl triflate in 81% yield.[186] Coupling of acceptor **50** with donor **52**, *via* the active intermediate formed upon treatment with DMTST, in the presence of 4 Å molecular sieves in dichloromethane, results in the formation of a complex mixture containing the desired fully protected disaccharide **62**, and the monoprotected disaccharides **63** and **64** (Scheme 17). Since disaccharides **63** and **64** cannot be isolated in pure form, the exact ratio of these compounds was not established.

Scheme 17. Attempted coupling of **50** and **52** with DMTST

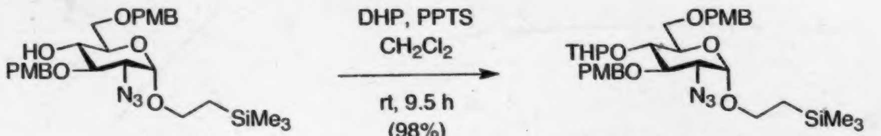


62: R = R' = PMB
63: R = H; R' = PMB
64: R = PMB; R' = H

To overcome these instability problems, different glycosylation conditions were explored. Unfortunately, reaction of donor **52** and acceptor **50** with *N*-iodosuccinimide and silver triflate as the promoters affords a complex mixture in which the major component seems to be, by mass analysis, a trisaccharide.

As the presence of *p*-methoxybenzyl groups is problematic for the glycosylation reaction, their replacement by acetyl groups, stable to the coupling conditions, was performed. The strategy chosen to achieve this modification first involves protection of the hydroxyl group of **50** (Scheme 18). Reaction of alcohol **50** with dihydropyran and pyridinium *p*-toluenesulfonate provides, in excellent yield (98%), the desired protected compound as mixture of diastereoisomers, which are separated by flash chromatography to give **65** and **66**. NMR analysis could not allow for unambiguous assignment of the relative stereochemistry for the tetrahydropyran moiety of diastereoisomers **65** and **66**.

Scheme 18. Protection of alcohol **50** by tetrahydropyran

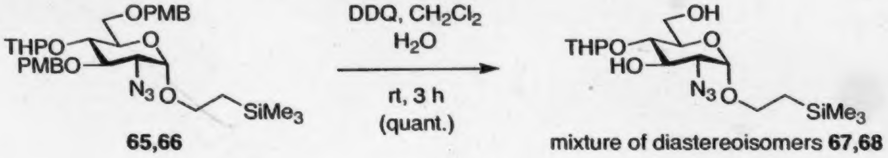


50

mixture of diastereoisomers **65,66**

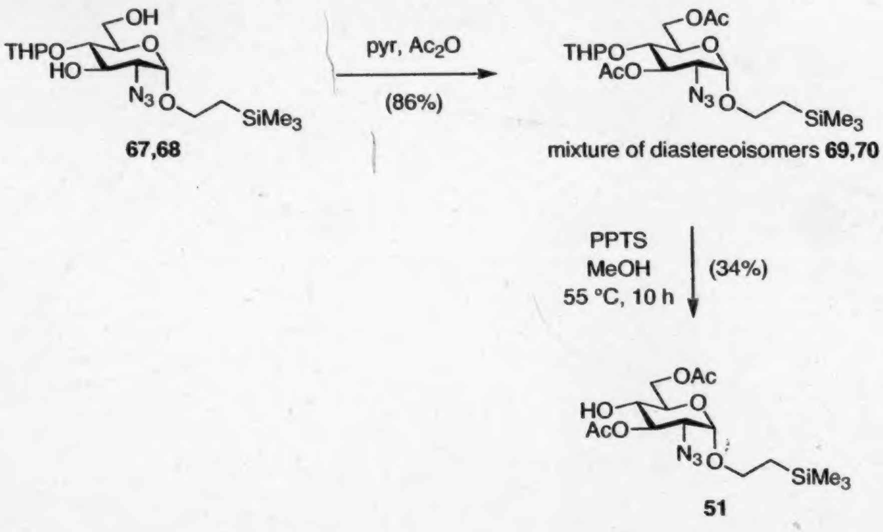
The next step involves selective removal of the *p*-methoxybenzyl protecting groups (Scheme 19). Although many reagents including ceric salts[187] and polymer supported-sulfonamides with trifluoromethanesulfonic acid[188] are reported in the literature to effect this transformation, only one example of oxidative removal of the *p*-methoxybenzyl ether in the presence of an azido functionality was found.[189] Treatment of the mixture of diastereoisomers **65** and **66** with DDQ in the presence of water in dichloromethane in the dark generates the desired alcohol as a mixture of diastereoisomers, which are separated by flash chromatography to provide **67** and **68**. The relative stereochemistry for the tetrahydropyran moiety of diastereoisomers **67** and **68** could not be unambiguously assigned by NMR spectroscopy.

Scheme 19. Oxidative removal of PMB from **65** and **66**


OPMB
THPO
PMBO
O
N3
O
SiMe3
65,66
DDQ, CH2Cl2
H2O
rt, 3 h
(quant.)
OH
THPO
HO
O
N3
O
SiMe3
mixture of diastereoisomers 67,68

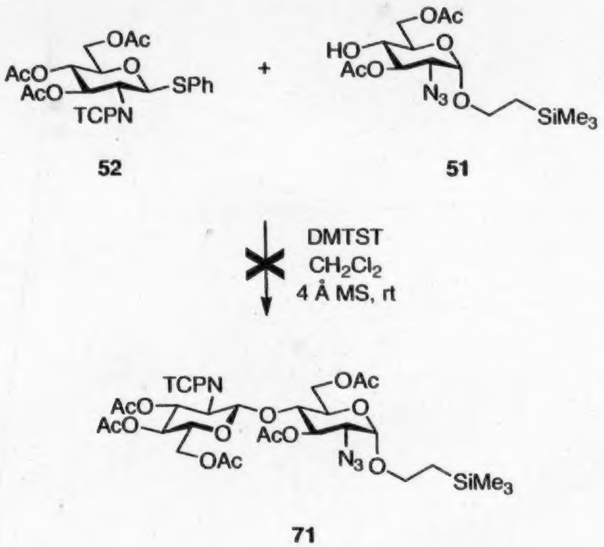
Reaction of diastereoisomers **67** and **68** with pyridine and acetic anhydride generates, in good yield (86%), the desired acetylated products as a mixture of diastereoisomers **69** and **70**, which cannot be separated (Scheme 20). Since removal of the tetrahydropyran protecting group follows, no effort was made to isolate **69** and **70** in pure form, and characterization of both compounds was performed as a mixture. Finally, removal of the tetrahydropyran protecting group using pyridinium *p*-toluenesulfonate in methanol furnishes the desired glycosyl acceptor **51** in 34% yield.

Scheme 20. Synthesis of glycosyl acceptor **51** from alcohols **67** and **68**


OH
THPO
HO
O
N3
O
SiMe3
67,68
pyr, Ac2O
(86%)
OAc
THPO
AcO
O
N3
O
SiMe3
mixture of diastereoisomers 69,70
PPTS
MeOH
55 °C, 10 h
(34%)
OAc
HO
AcO
O
N3
O
SiMe3
51

With the acetylated glycosyl acceptor **51** in hand, an attempt at its coupling with **52** was made (Scheme 21). Unfortunately, treatment of donor **52** and acceptor **51** with DMTST in dichloromethane containing 4 Å molecular sieves, fails to give any of the desired disaccharide **71**. The lack of success in the preparation of disaccharides **62** and **71** convinced us to abandon the preparation of type **B** targets and our efforts were directed toward the preparation of monosaccharides of type **C**.

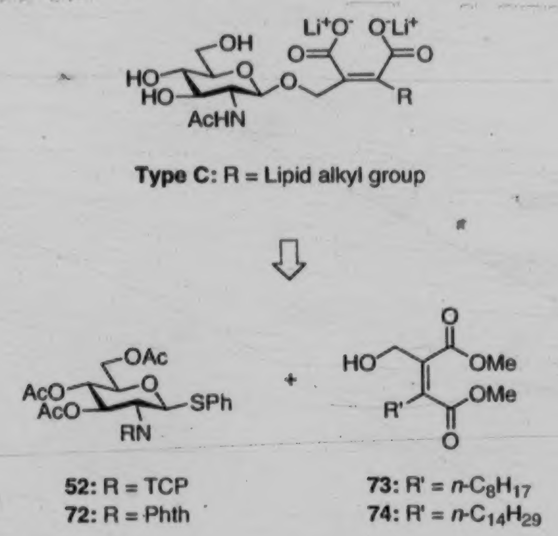
Scheme 21. Attempted glycosylation of **51** and **52** with DMTST



4. Synthetic Studies Toward Type C Targets: β-*O*-Glycosides

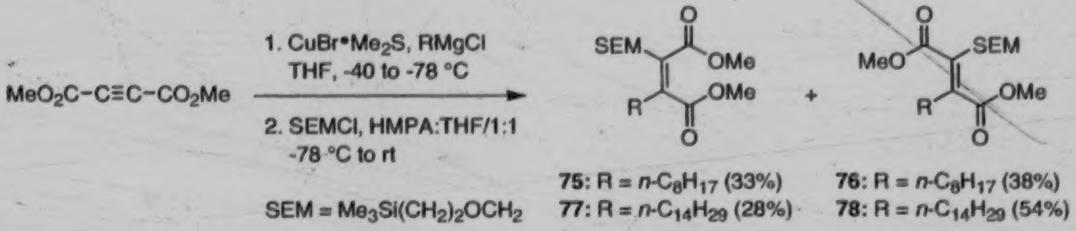
The ubiquitous presence of β-2-acetamido-2-deoxy sugars in nature[190] has led to the development of many synthetic strategies for their preparation. Based on the structure of lipid II, β-*O*-glycosides of type **C** have been designed to examine the importance of the configuration at the anomeric center for GTase inhibition. The retrosynthetic analysis for their preparation is based on the strategy depicted in Scheme 22. β-*O*-glycosides of type **C** could be accessible *via* an *O*-glycosylation reaction between glycosyl acceptors hydroxymethyl derivatives **73** and **74**, and suitably protected thioglycosides **52** or **72**.

Scheme 22. Retrosynthetic analysis for the preparation of type **C** targets: β-*O*-glycosides



The successful methodology previously utilized for the synthesis of chaetomellic acid A analogs **13** to **22** (Table 2) appears applicable for the preparation of key intermediates **73** and **74**. Even though previous attempts to introduce a hydroxymethyl functionality using this strategy failed to give the desired *Z* product in reasonable yield (Scheme 4), the addition of longer lipid chains seems to be the solution to the problems encountered earlier. Reaction of $CuBr{\bullet}Me_2S$, octylmagnesium chloride and DMAD in the presence of HMPA, followed by alkylation of the conjugate adduct with a trimethylsilylethoxymethyl chloride (SEMCl) gives a separable mixture of *Z* and *E* protected compounds **75** (33%) and **76** (38%) (Scheme 23). Similarly, reaction of tetradecylmagnesium chloride affords a separable mixture of *Z* and *E* protected compounds **77** (28%) and **78** (54%). The stereochemical assignment for these tetrasubstituted alkenes is based on NOE experiments. Even though the desired *Z* isomers **75** and **77** are the minor products formed during these reactions, enough material is produced to continue on the synthesis of hydroxymethyl derivatives **73** and **74**.

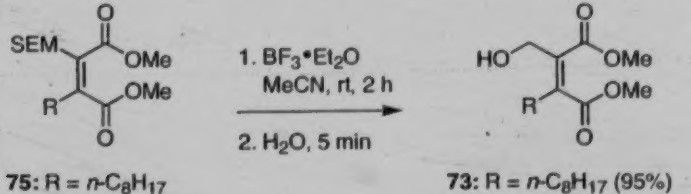
Scheme 23. Preparation of **75-78** by conjugate addition of SEMCl to DMAD


$MeO_2C-C\equiv C-CO_2Me$
1. CuBr•Me_2S, RMgCl
THF, -40 to -78 °C
2. SEMCl, HMPA:THF/1:1
-78 °C to rt
SEM = $Me_3Si(CH_2)_2OCH_2$
SEM
OMe
OMe
R
O
+
MeO
SEM
OMe
R
O

75: R = n-C_8H_{17} (33%)
77: R = n-$C_{14}H_{29}$ (28%)
76: R = n-C_8H_{17} (38%)
78: R = n-$C_{14}H_{29}$ (54%)

The use of the Lewis acid boron trifluoride etherate is an efficient method for the deprotection of (2-trimethylsilylethoxy)methyl compounds.[191] Removal of the trimethylsilylethyl group of **75** and **77** is achieved using BF_3•Et_2O in acetonitrile to afford the desired alcohols **73** and **74** in 95% yield (Scheme 24).

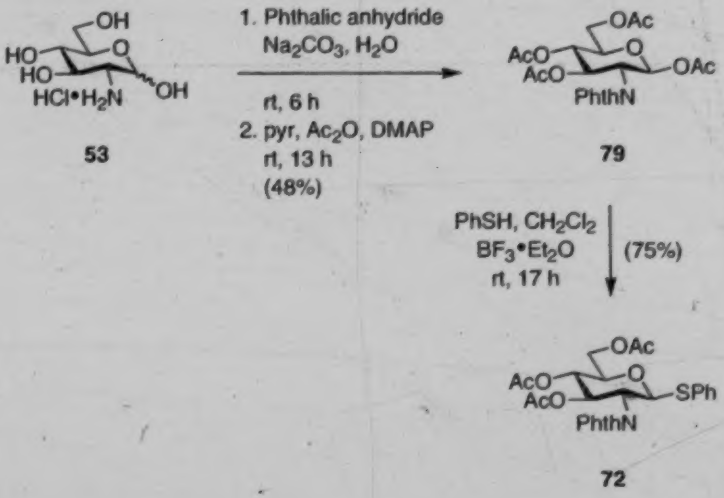
Scheme 24. Conversion of protected compounds **75** and **77** to alcohols **73** and **74**


SEM
OMe
OMe
R
O
1. BF_3•Et_2O
MeCN, rt, 2 h
2. H_2O, 5 min
HO
OMe
OMe
R
O

75: R = n-C_8H_{17}
77: R = n-$C_{14}H_{29}$
73: R = n-C_8H_{17} (95%)
74: R = n-$C_{14}H_{29}$ (95%)

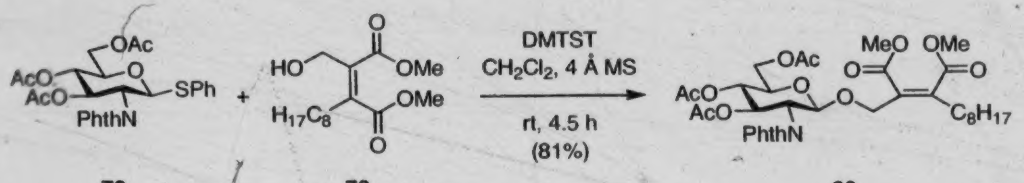
With enough of the required glycosyl acceptors **73** and **74** secured, the next step in the synthesis of β-*O*-glycosides of type **C** consists of preparing the correct glycosyl acceptors. In the past few years a large number of strategies has been successfully developed to obtain β-linked 2-acetamido-*D*-glucopyranosides. Among the reported amine protecting groups at position 2 required to form β-linked glycosides are found *N*-thiodiglycoloyl,[192] *N*-phthaloyl,[193] *N*-tetrachlorophthaloyl,[194] Troc,[195] methoxycarbonyl,[196] and acetyl.[197] Glycosyl donors containing *N*-acetyl moieties are rarely used as they tend to form stable oxazoline intermediates that are unreactive with many acceptors.[198] The most widely used amine protecting group is the phthalimido moiety first introduced by Lemieux and co-workers.[193] The preparation of glycosyl donor **72** containing a phthalimido participating group is outlined in Scheme 25. Treatment of *D*-glucosamine hydrochloride (**53**) with phthalic anhydride and aqueous sodium carbonate affords 2-phthalimido-2-deoxy-*D*-glucose, which is immediately acetylated using pyridine and acetic anhydride in the presence of 4-(dimethylamino)pyridine to give **79** in 48% yield. Reaction of **79** with thiophenol and $BF_3 \cdot Et_2O$ gives the desired thioglucoside **72** in 75% yield.

Scheme 25. Synthesis of glycosyl donor **72** from *D*-glucosamine hydrochloride (**53**)


OH
HO
HO
O
HCl•H₂N
OH
53
1. Phthalic anhydride
Na₂CO₃, H₂O
rt, 6 h
2. pyr, Ac₂O, DMAP
rt, 13 h
(48%)
OAc
AcO
AcO
O
OAc
PhthN
79
PhSH, CH₂Cl₂
BF₃•Et₂O
rt, 17 h
(75%)
OAc
AcO
AcO
O
SPh
PhthN
72

Coupling of glycosyl donor **72** and acceptor **73** using DMTST[186] as a promoter with 4 Å molecular sieves in dichloromethane produces the desired β-*O*-glycoside **80** in 81% yield.

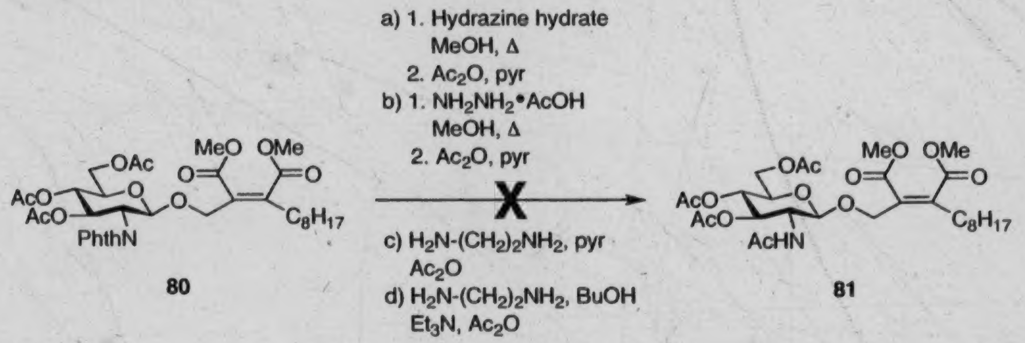
Scheme 26. Synthesis of **80** by glycosylation reaction between **72** and **73**



72 **73** **80**

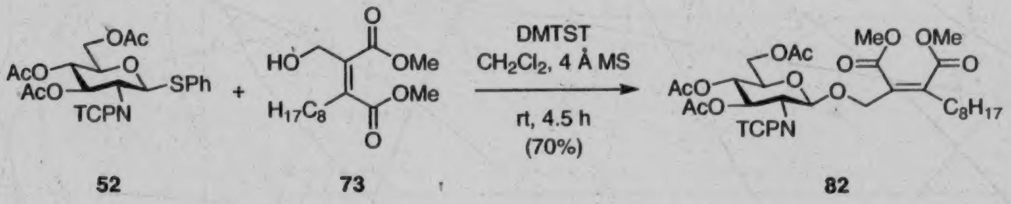
With the glycosylation reaction achieved, deprotection of the phthalimido moiety followed by protection of the obtained amino group is required to furnish the desired acetamido functionality. Several reagents have been reported in the literature to effect this transformation including hydrazine hydrate,[199] hydrazine acetate,[200] and ethylene diamine. However, attempts to synthesize the desired acetamido protected compound **81** using these various methods do not give the desired product (Scheme 27). With this failure to successfully deprotect phthalimido **80** an alternative protecting group at position 2 was investigated.

Scheme 27. Attempted removal of the phthalimido moiety of **80**


a) 1. Hydrazine hydrate
MeOH, Δ
2. Ac2O, pyr
b) 1. NH2NH2•AcOH
MeOH, Δ
2. Ac2O, pyr
c) H2N-(CH2)2NH2, pyr
Ac2O
d) H2N-(CH2)2NH2, BuOH
Et3N, Ac2O
OAc
AcO
AcO
PhthN
MeO
OMe
C8H17
AcHN
80
81

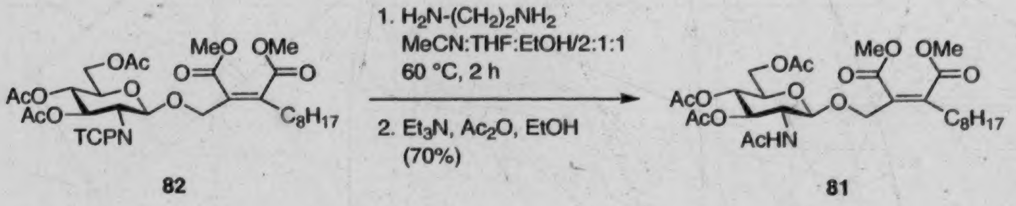
The conditions required to remove the phthalimido group are usually quite harsh. It is known that the *N*-tetrachlorophthaloyl protecting group can be removed under milder conditions. With the success of the formation of **80** from **72** and **73** (Scheme 26), the analogous synthesis was repeated with the previously synthesized tetrachlorophthalimido analog **52**, as outlined in Scheme 28. Glycosylation in dichloromethane containing 4 Å molecular sieves with DMTST activation and the glycosyl donor **52** (Scheme 16) and acceptor **73** (Scheme 24) gives the desired coupled product **82** in 70% yield.

Scheme 28. Synthesis of **82** by glycosylation reaction between **52** and **73**


OAc
AcO
AcO
TCPN
SPh
+
HO
OMe
OMe
H17C8
DMTST
CH2Cl2, 4 Å MS
rt, 4.5 h
(70%)
MeO
OMe
C8H17
52
73
82

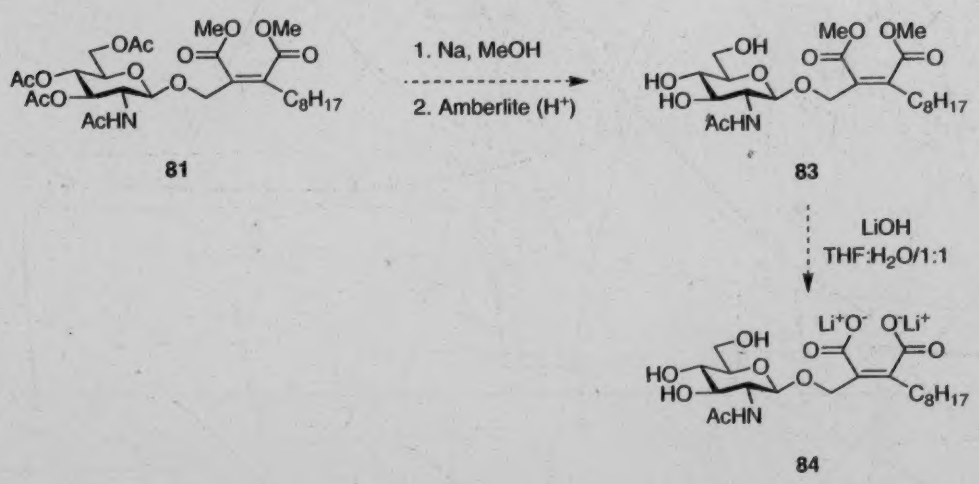
Several reaction conditions have been reported in the literature to effect the transformation of a tetrachlorophthalimido group to an acetamido functionality.[201-204] It has been suggested that the correct choice of solvent is crucial to realize this transformation.[205] As depicted in Scheme 29, upon treatment with ethylenediamine in a 2:1:1/acetonitrile:tetrahydrofuran:ethanol mixture of solvents tetrachlorophthalimido **82** is transformed into the corresponding amino compound, which is immediately reacted with acetic anhydride and triethylamine in ethanol to give the desired acetamide **81** in 70% yield.

Scheme 29. Transformation of tetrachlorophthalimide **82** into acetamide **81**


OAc
AcO
AcO
O
TCPN
O
MeO
OMe
O
O
C8H17
82
1. H2N-(CH2)2NH2
MeCN:THF:EtOH/2:1:1
60 °C, 2 h
2. Et3N, Ac2O, EtOH
(70%)
OAc
AcO
AcO
O
AcHN
O
MeO
OMe
O
O
C8H17
81

Removal of the *O*-acetyl groups using sodium methoxide followed by hydrolysis of the esters with lithium hydroxide should afford the type **C** target **84** (Scheme 30). These reactions are currently being performed.

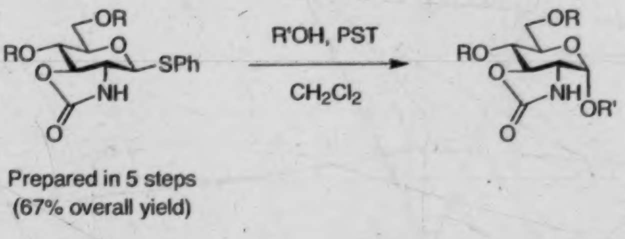
Scheme 30. Synthesis of β-*O*-glycoside of type **C 84**


OAc
AcO
AcO
O
AcHN
O
MeO
OMe
O
O
C8H17
81
1. Na, MeOH
2. Amberlite (H+)
OH
HO
HO
O
AcHN
O
MeO
OMe
O
O
C8H17
83
LiOH
THF:H2O/1:1
OH
HO
HO
O
AcHN
O
Li+O-
O-Li+
O
O
C8H17
84

5. Synthetic Studies Toward Type C Targets: α-*O*-Glycosides

To achieve 1,2-*cis*-glycosylation a non-participating group at position C-2 is usually required. While there are a number of strategies to obtain β-linked 2-acetamido-*D*-glycopyranosides, the more difficult formation of α-linked 2-acetamido-*D*-glycopyranosides relies almost exclusively on using 2-azido-glycosyl donors.[206,207] Recently, an alternative method involving oxazolidinone protected 2-amino-2-deoxy-*D*-glucose derivatives has also been shown to produce α-linked 2-amino-glycosides (Figure 10).[208]

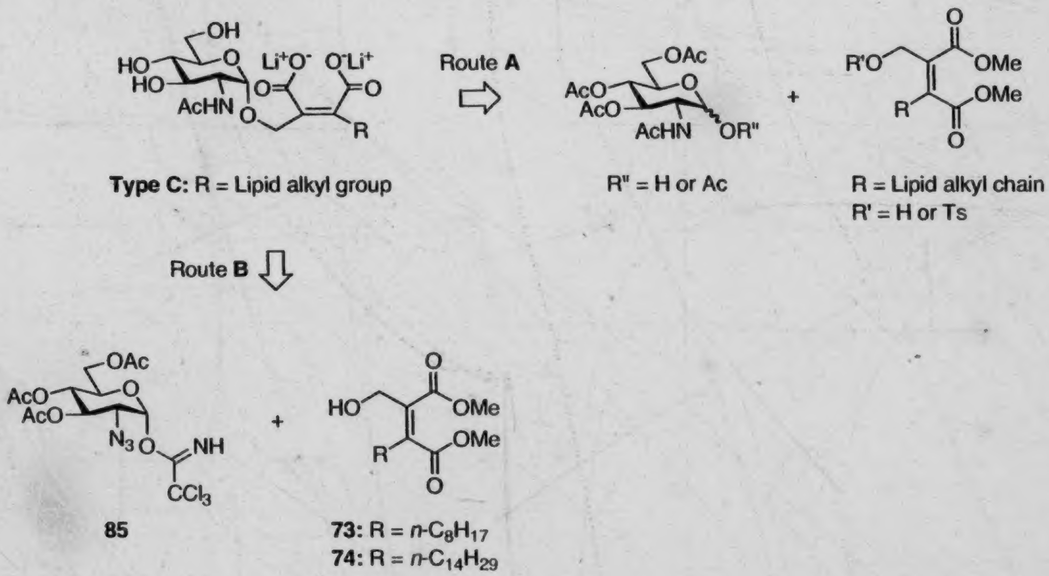
Figure 10. Non-participating oxazolidinone ring affords α-glycosides



Retrosynthetic analysis of type **C** targets with a α-configuration at the anomeric center indicates that their preparation can be realized *via* two routes **A** and **B** (Scheme 31). Route **A** provides the desired 2-acetamido-α-*D*-glucopyranosides directly from 2-acetamido donors. Although a literature survey reveals a single precedent for such a transformation,[209] the idea seems attractive since very few steps are involved in the preparation of the glycosyl donors and acceptors. Route **B** that involve the more traditional use of 2-azido-2-deoxy sugars affords the desired α-*O*-glycosides of type **C** from α-trichloroacetimidate **85** and the already available aglycons **73** and **74** (Scheme

24). Both routes outlined in Scheme 31 were attempted in order to produce the targeted molecules.

Scheme 31. Retrosynthetic analysis for the preparation of type **C** targets: α-*O*-glycosides

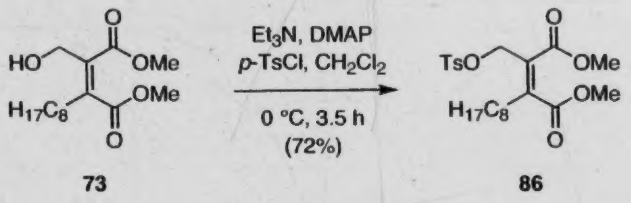

Route **A**
Route **B**
Type C: R = Lipid alkyl group
R" = H or Ac
R = Lipid alkyl chain
R' = H or Ts
85
73: R = n-C8H17
74: R = n-C14H29

5.1. Route A: Attempted Synthesis of α-Linked Derivatives from Precursor with *N*-Acetyl Protecting Group

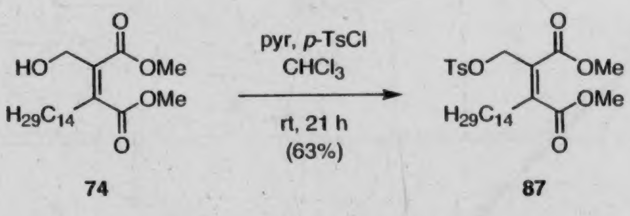
2-Acetamido sugars are known to produce β-linkages through neighboring group participation during glycosylation reactions. Although the direct introduction of a linker on 2-acetamido sugars in a 1,2-*cis* fashion is not favored, previous studies indicate that such a transformation can occur between the sodium salt of 3,4,6-tri-*O*-acetyl-2-acetamido-2-deoxy-*D*-glucopyranoside and β-tosyloxy acrolein.[209] In the hope of directly synthesizing α-*O*-linked derivatives of *N*-acetylglucosamine (type **C** targets), tosylates **86**

(72%) and **87** (63%) are prepared as glycosyl acceptors by treatment of alcohols **73** and **74** with *p*-toluenesulfonyl chloride under basic conditions (Schemes 29 and 30).

Scheme 32. Synthesis of tosylate **86** from alcohol **73**

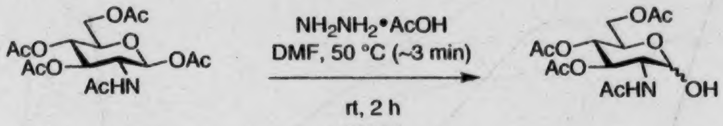

HO
OMe
OMe
$H_{17}C_8$
O
O
73
Et_3N, DMAP
p-TsCl, CH_2Cl_2
0 °C, 3.5 h
(72%)
TsO
OMe
OMe
$H_{17}C_8$
O
O
86

Scheme 33. Synthesis of tosylate **87** from alcohol **74**


HO
OMe
OMe
$H_{29}C_{14}$
O
O
74
pyr, p-TsCl
$CHCl_3$
rt, 21 h
(63%)
TsO
OMe
OMe
$H_{29}C_{14}$
O
O
87

The next step involves the preparation of glycosyl donor **89** from commercially available 1,3,4,6-tetra-*O*-acetyl-2-acetamido-2-deoxy-β-*D*-glycopyranose (**88**) (Scheme 34). Various reagents including benzyl amine in THF (85%),[210,211] ammonia in MeCN:THF:MeOH,[212] lipase from *Aspergillus niger* AP_6 Amano (93%),[209,213] sodium methanolate (86%),[214] and hydrazine acetate in DMF,[215] have been reported for the 1-*O*-deacetylation of compound **88**. Treatment of **88** with hydrazine acetate using a modified literature procedure,[215] gives the glycosyl donor **89** in 82% yield. The desired product is obtained in lower yields when other methods are used.

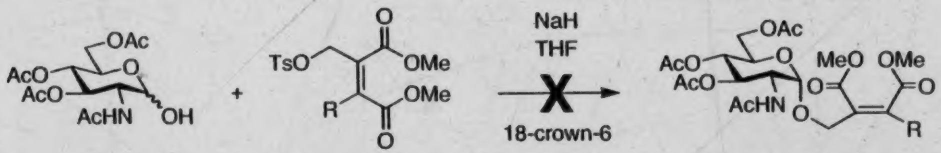
Scheme 34. Synthesis of reducing sugar **89** by 1-*O*-deacetylation of **88**


OAc
AcO
AcO
OAc
AcHN
O
NH2NH2•AcOH
DMF, 50 °C (~3 min)
rt, 2 h
(82%)
OAc
AcO
AcO
AcHN
OH

88 **89**

With both the glycosyl acceptors and donor in hand, we investigated the glycosylation reaction. Unfortunately, treatment of alcohol **89** with tosylates **86** or **87** in the presence of NaH and a small amount of 18-crown-6 (1 mol%) does not yield any of the desired α-*O*-glycosides (Scheme 35).

Scheme 35. Attempted synthesis of α-monosaccharides **90** and **91** using NaH


OAc
AcO
AcO
AcHN
OH
+
TsO
O
OMe
OMe
R
O
NaH
THF
18-crown-6
OAc
AcO
AcO
AcHN
MeO
OMe
O
R

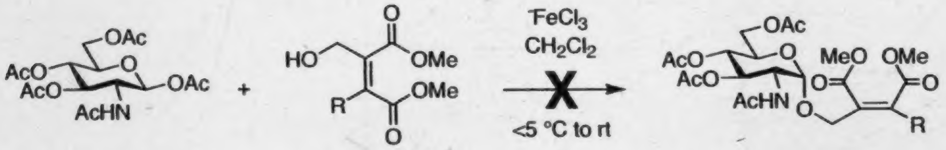
89

86: R = n-C_8H_{17}
87: R = n-$C_{14}H_{29}$

90: R = n-C_8H_{17}
91: R = n-$C_{14}H_{29}$

Another strategy for the stereoselective α-glycosylation of peracetylated glycosides has also been reported in the literature.[216] This methodology involves the use of $FeCl_3$ as a Lewis acid catalyst. Having all the required reactants already synthesized for this type of reaction, we thought that this methodology could be extended to the preparation of **90** and **91** (Scheme 36). Unfortunately, attempts at coupling commercially available 1,3,4,6-tetra-*O*-acetyl-2-acetamido-2-deoxy-β-*D*-glycopyranose (**88**) with alcohols **73** and **74** (Scheme 24) yield none of the desired α-*O*-glycosides **90** and **91**. Therefore this strategy was abandoned and our efforts were directed toward the use of more conventional methods involving 2-azido sugars for the formation of the desired type **C** targets.

Scheme 36. Attempted synthesis of α-*O*-glycosides **90** and **91** using $FeCl_3$



88

73: R = n-C_8H_{17}
74: R = n-$C_{14}H_{29}$

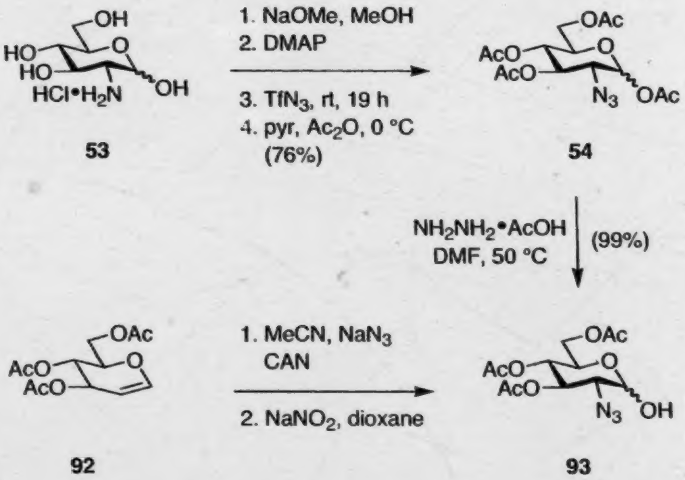
90: R = n-C_8H_{17}
91: R = n-$C_{14}H_{29}$

5.2. Route B: Preparation of Type C Targets Using 2-Azido Sugars

The significance of *O*-glycosyl trichloroacetimidates for the formation of α-*O*-glycosides from 2-azido sugars has been widely investigated. It is known that upon treatment with acid they produce the desired α-linkages. The synthesis of targeted α-*O*-monosaccharides of type **C** uses the trichloroacetimidate method and begins with the preparation of reducing sugar **93** (Scheme 37). Glycosyl donor **93** can be prepared *via* two different approaches. Based on a methodology described by Kinzy and Schmidt,[174] compound **93** is directly obtained by treatment of 3,4,6-tri-*O*-acetyl-*D*-glucal (**92**) with sodium azide, ceric ammonium nitrate and sodium nitrite. However, isolation of compound **93** thus formed is very difficult. The alternative two-step procedure outlined in Scheme 37 provides the desired reducing sugar **93** (75% overall yield) in better yield. It also has the advantage of simpler purification. As previously described (Scheme 13), azide **54** is produced by treatment of *D*-glycosamine hydrochloride (**53**) with sodium methoxide, 4-(dimethylamino)pyridine and freshly prepared triflic azide, in 76% yield.

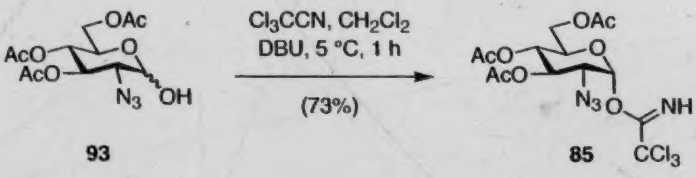
Azide **54** is easily converted to the corresponding alcohol **93** in 99% yield using hydrazine acetate in DMF.

Scheme 37. Synthesis of reducing sugar **93** *via* two different approaches


OH
HO
HO
O
HCl•H2N
OH
53
1. NaOMe, MeOH
2. DMAP
3. TfN3, rt, 19 h
4. pyr, Ac2O, 0 °C
(76%)
OAc
AcO
AcO
O
N3
OAc
54
NH2NH2•AcOH
DMF, 50 °C
(99%)
OAc
AcO
AcO
O
92
1. MeCN, NaN3
CAN
2. NaNO2, dioxane
OAc
AcO
AcO
O
N3
OH
93

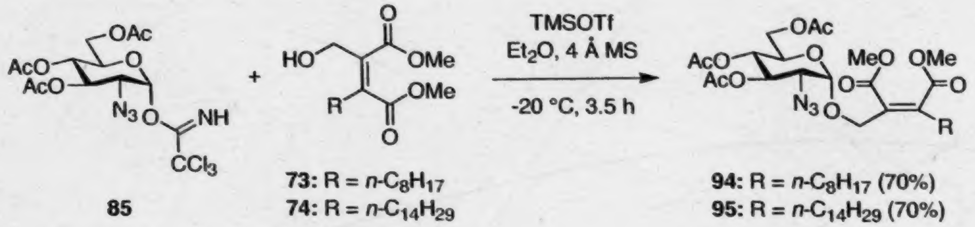
Subsequent imidation of **93** utilizing trichloroacetonitrile and DBU in dichloromethane gives the desired α-trichloroacetimidate **85** in 73% yield (Scheme 38). Compound **85** is not very stable and is immediately used after purification for the next reaction. Other bases, such as potassium carbonate, can also be used for the synthesis of **85**.

Scheme 38. Synthesis of α-trichloroacetimidate **85** from alcohol **93**


OAc
AcO
AcO
O
N3
OH
93
Cl3CCN, CH2Cl2
DBU, 5 °C, 1 h
(73%)
OAc
AcO
AcO
O
N3
O
NH
CCl3
85

The glycosylation of trichloroacetimidate can be promoted by various reagents including $BF_3 \cdot Et_2O$ and trimethylsilyl triflate. Reaction of α-trichloroacetimidate **85** with alcohols **73** and **74** in the presence of trimethylsilyl triflate and 4 Å molecular sieves in diethyl ether produces the desired α-*O*-glycosides **94** and **95** in 70% yield.

Scheme 39. Synthesis of **94** and **95** by glycosylation reaction between **85** and **73** and **74**


AcO
AcO
OAc
O
N3
O
NH
CCl3
HO
O
OMe
OMe
R
O
+
TMSOTf
Et2O, 4 Å MS
-20 °C, 3.5 h
AcO
AcO
OAc
O
N3
O
MeO
OMe
O
O
R

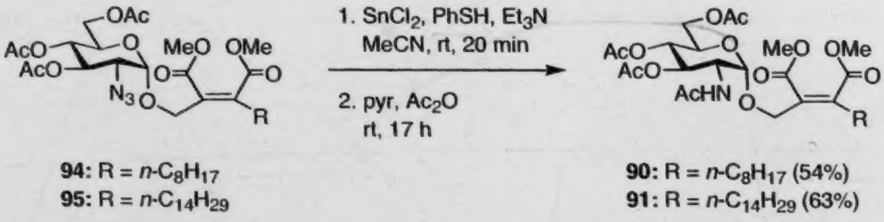
85

73: R = *n*-C_8H_{17}
74: R = *n*-$C_{14}H_{29}$

94: R = *n*-C_8H_{17} (70%)
95: R = *n*-$C_{14}H_{29}$ (70%)

With the α-glycosidic linkage in place, the next transformation requires conversion of the azido group to an acetamido moiety (Scheme 40). Reaction of azides **94** and **95** with tin chloride, thiophenol and triethylamine in acetonitrile affords the corresponding amino compounds which are immediately treated with pyridine and acetic anhydride to produce the desired acetamides **90** and **91** in 54% and 63% yield, respectively.

Scheme 40. Transformation of azides **94** and **95** into acetamides **90** and **91**

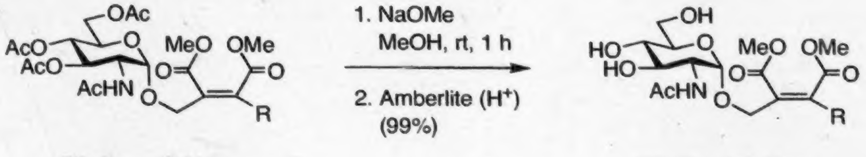

AcO
AcO
OAc
O
N3
O
MeO
OMe
O
O
R
1. SnCl2, PhSH, Et3N
MeCN, rt, 20 min
2. pyr, Ac2O
rt, 17 h
AcO
AcO
OAc
O
AcHN
O
MeO
OMe
O
O
R

94: R = *n*-C_8H_{17}
95: R = *n*-$C_{14}H_{29}$

90: R = *n*-C_8H_{17} (54%)
91: R = *n*-$C_{14}H_{29}$ (63%)

Deprotection of the hydroxyl functionality of **90** and **91** using methanolic sodium methoxide affords diesters **96** and **97** in 99% yield (Scheme 41).

Scheme 41. Deacetylation of **90** and **91**

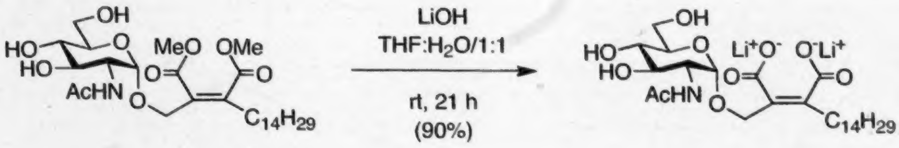

OAc
AcO
AcO
O
MeO
OMe
O
O
AcHN
O
R
1. NaOMe
MeOH, rt, 1 h
2. Amberlite (H⁺)
(99%)
OH
HO
HO
R

90: R = n-C_8H_{17}
91: R = n-$C_{14}H_{29}$

96: R = n-C_8H_{17}
97: R = n-$C_{14}H_{29}$

Finally, hydrolysis of the methyl esters of **97** with lithium hydroxide furnishes the desired type **C** derivative **98** (Scheme 42).

Scheme 42. Synthesis of α-*O*-glycoside of type **C** by ester hydrolysis of **97**


OH
HO
HO
O
MeO
OMe
O
O
AcHN
O
C14H29
LiOH
THF:H2O/1:1
rt, 21 h
(90%)
Li⁺O⁻
O⁻Li⁺

97

98

Three types of lipid II analogs were designed to inhibit peptidoglycan biosynthesis. The preparation of type **A** and **B** targets failed. The successfully prepared chaetomellic acid A analogs **13-16** and type **C** compound **98** with the α-configuration at the anomeric center are currently being tested by Suzanne Walker (Department of Chemistry, Princeton University) as peptidoglycan GTase inhibitors.

CHAPTER 2. Bacteriocins of Lactic Acid Bacteria (LAB) as Inhibitors of Peptidoglycan Biosynthesis

INTRODUCTION

Bacteriocins are peptides produced by bacteria that display antimicrobial properties against other bacteria, often closely related to the producer strain.[217,218] The first bacteriocin was discovered in 1925,[219] and in the past twenty years there has been an increasing interest in such compounds as possible preservative agents for food, and as potential supplements or replacements for currently used antibiotics. The ribosomal production of these antimicrobial peptides by Gram-positive bacteria, especially lactic acid bacteria, as a defense mechanism against other organisms is well documented and represents an area of intensive research.[220,221] Lactic acid bacteria (LAB) are a heterogeneous group of Gram-positive, anaerobic, nonsporulating coccus- or rod-shaped bacteria with a DNA base composition of less than 50 mol% G+C. LAB are found in a wide variety of habitats including foods (*e.g.* dairy products, fermented vegetables and meats, sourdough bread, and beverages), silage, sewage, plants, and gastrointestinal, respiratory and urogenital tracts of humans and animals.[222] The competitive environments in which many Gram-positive bacteria find themselves has probably led to their ability to generate novel bacteriocins, often resulting in peptides bearing unique structural features and varied modes of action. Bacteriocins of LAB are a heterogeneous group of bacterial antagonists that vary from small peptides (few thousand Da) to large proteins that might contain lipid or carbohydrate moieties. Bacteriocins have been divided into four main categories (Table 4).[223-225]

Table 4. Classification of bacteriocins from Gram-positive bacteria[223-225]

Class	*Characteristics and subclasses*
I. Lantibiotics:	*Ribosomally produced peptides that undergo extensive posttranslational modification* *Small (<5 kDa) peptides containing lanthionine and β-methyllanthionine* Ia. Flexible molecules compared to Ib Ib. Globular peptides with no net charge or net negative charge
II. Nonlantibiotics:[a]	*Low-molecular-weight (<10 kDa), heat stable peptides* *Formed exclusively from unmodified amino acids* *Ribosomally synthesized as inactive prepeptides that get activated by posttranslational cleavage of the N-terminal leader peptide* IIa. Anti-listerial single peptides that contain YGNGVXC amino acid motif near their N termini IIb. Two-peptide bacteriocins IIc. Bacteriocins produced by the cell's general *sec*-pathway[224]
III. Nonlantibiotics:	*High-molecular-weight (>30 kDa), heat labile proteins*
IV[b]	*Complex bacteriocins carrying lipid or carbohydrate moieties*

[a] Nonlantibiotics have recently been re-subdivided by van Belkum according to the bacteriocins that they resemble rather than to their method of excretion from the cell.[223]

[b] This category has been excluded from Nes' classification.[224]

Among the known bacteriocins, two distinct families have emerged: the nonlantibiotics and the lantibiotics.[226,227] The latter contain the unusual amino acids lanthionine and β-methyllanthionine as part of additional intramolecular rings, and often possess other modified residues, frequently dehydro amino acids, which are formed after ribosomal translation. Most bacteriocins that have been studied belong to classes I and II. Both types of bacteriocins are most frequently found as single active peptides, but the class of two-peptide systems wherein both components are required for full activity is growing quickly. We recently published a review that addresses only those two-peptide systems wherein one or both of the two peptides is inactive alone but in combination an enhanced antimicrobial effect is observed.[228] Since 1994 several review articles have been published on bacteriocins produced by Gram-positive bacteria giving detailed information on their biosynthesis,[224,229-232] structure and mechanism of action,[223,233-240] purification,[241,242] biological activity,[243,244] and applications.[245-262] All the bacteriocins examined in this work belong to class II.

1. Biosynthesis of Class II Bacteriocins

Usually ribosomally formed as prepeptides in the cell, the mature bacteriocins (both one and two component systems) are generated by processing during export. This is generally accomplished via the enzymatic removal of a N-terminal leader peptide either by a *sec*-dependent pathway, or more often at a specific double-glycine position (-2, -1) in the prepeptide concomitant with externalization by a dedicated ABC-transporter.[263-266] With the exception of disulfide bridge formation, posttranslational modifications are rare among the nonlantibiotic bacteriocins, and sequence determination of the peptides is generally facile *via* automated Edman degradation technology.

At least four different genes are required to achieve the production of bacteriocins by Gram-positive bacteria: (i) a structural gene encoding the prepeptide; (ii) a dedicated immunity gene; (iii) a gene encoding a dedicated ABC-transporter; and (iv) a gene encoding an accessory protein necessary for the externalization of the bacteriocin.[224] These genes are encoded either on chromosome, on plasmids or on both.

The structural gene encodes the biologically inactive[267] prebacteriocin that contains a N-terminus extension or leader sequence which is subsequently cleaved at the specific processing site to form the active antimicrobial molecule. The amino acid sequences (14-30 residues) of double-glycine leader peptides usually contain hydrophobic residues at positions -4, -7, -12 and -15, and hydrophilic residues at positions -5, -6 and -11. Their secondary structures are predicted to be α-helical. Bacteriocins generated by a *sec*-dependent pathway contain an N-terminal signal peptide which is normally longer than double-glycine leader peptides and have a hydrophobic central domain, a neutral C-terminal portion, and a positively charged N-terminal domain.[268]

The immunity gene, almost always located downstream of the structural gene to which it is normally linked, produces proteins that protect the bacteriocin producers against their own bacteriocin and renders them partially immune to other class IIa bacteriocins.[269] Immunity proteins, which vary in length from 51 up to 154 amino acid residues, usually share little or no sequence homology.[270] However, they are cationic and mostly hydrophobic molecules. Secondary structure predictions[271,272] indicate that immunity proteins are largely α-helical. Studies on immunity proteins MesI[273] and CbiB2,[274] found for the most part in the cytoplasm with a small portion in the cytoplasmic membrane, suggest that the mechanism by which they inactivate bacteriocins is indirect and proceeds *via* a membrane-bound protein. This mechanism of action remains to be proved.

The ABC-transporter and accessory protein, constituents of the dedicated bacteriocin transport apparatus, are membrane-bound proteins that are required for transmembrane translocation of class II bacteriocins. These ABC-transporters generally contain three domains: a N-terminal cysteine protease domain[275] (150 amino acids with a cysteine and a histidine conserved motifs), a hydrophobic membrane-spanning domain, and a C-terminal ATP-binding domain.[224] For certain class II bacteriocins, secretion can occur by using double-glycine leader peptides or the signal peptide of divergicin A.[276-280]

Accessory proteins are thought to facilitate the membrane translocation and the processing of the leader peptide. Both their C- and N-terminal domains are hydrophobic. Accessory proteins are required for successful externalization of bacteriocins.

2. Bacteriocins as Inhibitors of Peptidoglycan Biosynthesis

Several studies have been conducted on the mode of action of bacteriocins of LAB.[281] The action of these small cationic peptides is directed primarily against bacteria of Gram-positive species. Many bacteriocins appear to elicit their lethal effects by permeabilizing the cell membrane of target organisms, in certain cases by targeting intermediates of cell wall biosynthesis[282,283] or possibly proteins of sugar phosphotransferase systems.[284,285] Lantibiotics such as nisin (**99**),[236,239,282,286,287] mersacidin (**100**),[288-290] and actagardine (**101**)[291] (Figure 11) have been found to require lipid II as a receptor or docking molecule. These molecules can inhibit peptidoglycan formation and thereby kill bacterial cells.[283,292] However, type Ia lantibiotics such as nisin (**99**), epidermin and Pep5 are flexible, elongated, amphipathic molecules whose primary mode of action is the formation of pores in the bacterial cytoplasmic membrane.[293] The recently proposed mechanism of action of nisin is depicted in Figure 12.[282] Nisin first binds to the carbohydrate moiety of lipid II oriented toward the outside of the cell in a 1:1 ratio. A negatively charged surface is not essential for binding, but the N-terminal region of nisin is essential for lipid II recognition and prevention of peptidoglycan biosynthesis. The C-terminal region of nisin and a flexible "hinge" region then translocate across the cytoplasmic membrane to allow highly specific pore formation resulting in the release of monovalent cations and ATP.

Figure 11. Primary structure of bacteriocins that inhibit peptidoglycan formation

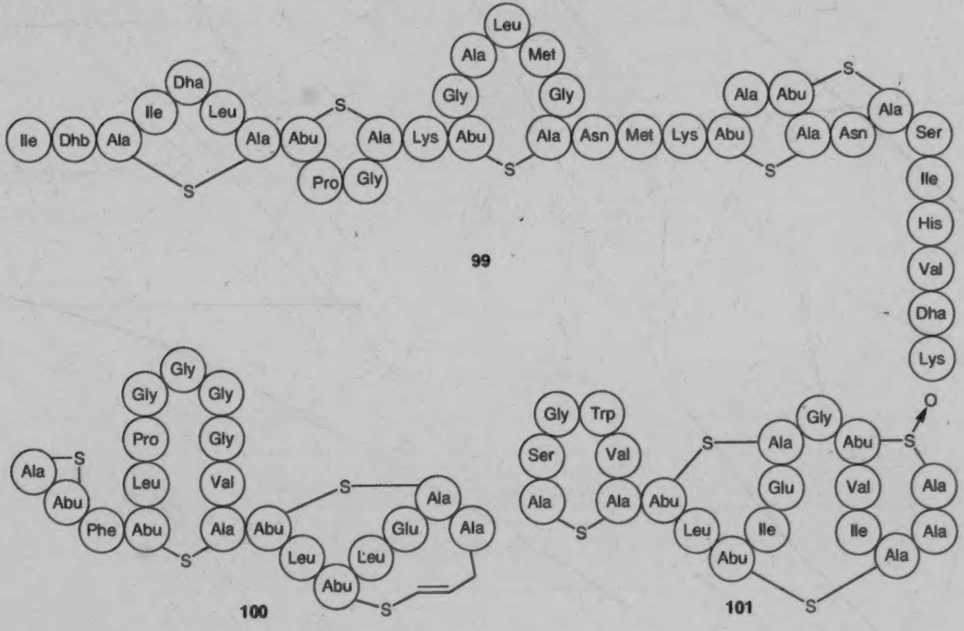
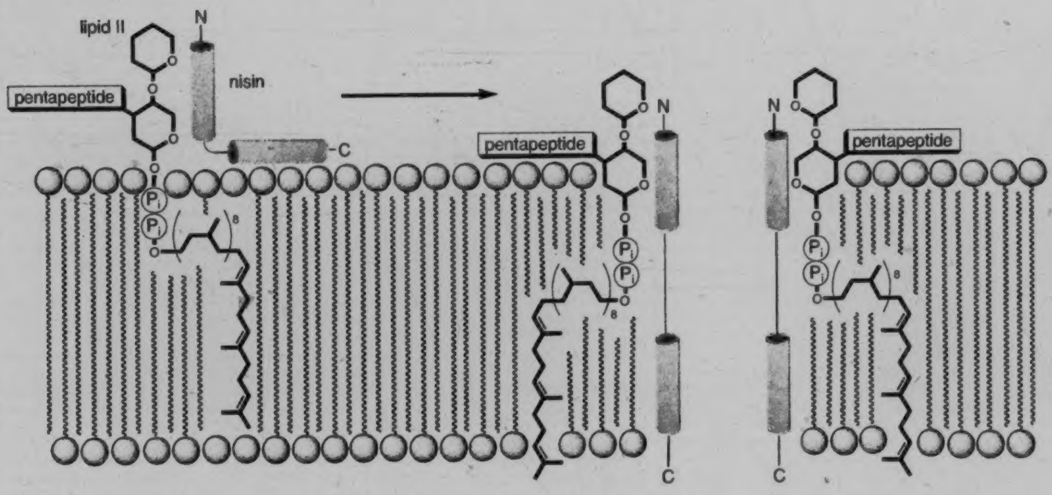

Ile
Dhb
Ala
Ile
Dha
Leu
Ala
S
Abu
S
Pro
Gly
Ala
Lys
Gly
Ala
Leu
Met
Gly
Abu
S
Ala
Asn
Met
Lys
Abu
Ala
Abu
S
Ala
Asn
S
Ala
Ser
Ile
His
Val
Dha
Lys
99
Ala
S
Abu
Phe
Abu
Leu
Pro
Gly
Gly
Gly
Gly
Val
Ala
S
Abu
Leu
Abu
Leu
Glu
S
Ala
Ala
S
100
Gly
Trp
Ser
Val
Ala
Ala
S
Abu
Leu
Abu
S
Ala
Glu
Ile
Gly
Abu
Val
Ile
Ala
S
O
Ala
Ala
S
101

Figure 12. Binding of nisin to lipid II causes pore formation


lipid II
pentapeptide
N
nisin
C
P
P
8
pentapeptide
N
C
N
pentapeptide
C

Type Ib lantibiotics such as mersasidin (**100**) and actagardine (**101**) have a rigid globular shape and inhibit GTases by forming a tight complex with their membrane-bound substrates.[289] The action of mersasidin at the transglycosylation level by interaction with lipid II occurs *via* a binding site that is not targeted by any currently used antibiotic.

3. Bacteriocins of LAB as Food Preservatives

Listeria monocytogenes represents a significant health threat as an infectious agent in foods such as meat, dairy and egg products, and vegetables.[294,295] The principal physical, chemical, enzymatic and microbiological reactions responsible for other forms of food deterioration are also well known.[262] Various preservation techniques are used to avoid different forms of spoilage and food poisoning, including reduction of temperature, water activity, and pH, as well as addition of preservatives such as antimycotic (*e.g.* natamycin), inorganic (sulphite, nitrite) and organic compounds (propionate, sorbate, benzoate). Other technologies for food preservation include pasteurization and sterilization by heating, as well as packaging and aseptic processing to restrict access of microorganisms to products. With the increasing demand for more natural and microbiologically safe food products, there is a need for new preservation techniques. Among the emerging conservation technologies is the use of natural additives such as egg white lysozyme and bacteriocins of lactic acid bacteria (*e.g.* nisin) to control the growth and survival of undesirable microorganisms.[296,297] Nisin has been employed for over 50 years and is currently approved in more than 80 countries for use in cheese, liquid egg products, canned vegetables, diverse pasteurized dairy, and salad dressing. It is the only purified antibiotic peptide that has been licensed for utilization as a food preservative by the U.S. Food and Drug Administration.[298] However, many examples of inclusion of bacteriocin-producing starter cultures in food fermentation have been reported in the literature.[245,248,299-301]

4. Scope of the Project

One of the greatest challenges in bacteriocin research is the understanding of the relationships between structure and function of such compounds. Two different approaches for the study of bacteriocins as potential inhibitors of peptidoglycan biosynthesis were undertaken. The first goal of our research was to isolate new bacteriocins from various strains of *Carnobacterium piscicola* (307 and 682). The purification procedures, the genetic characterization and the description of the known (piscicolin 126 and carnobacteriocins B1 and BM1) and new bacteriocins isolated are presented in the first part of this chapter.

Since knowledge of the three-dimensional structure of bacteriocins can provide a basis for detailed studies on structure-activity relationships[302] and mechanism of action, our group has employed nuclear magnetic resonance (NMR) spectroscopy to determine structures of single-peptide type IIa bacteriocins such as leucocin A (LeuA)[303] and carnobacteriocin B2.[304] In the second part of the present study, as a prelude to NMR examination of two-peptide bacteriocin interactions, we characterize the two peptides of brochocin C (BrcC) produced by separate clones of BrcA and BrcB in *Carnobacterium piscicola* LV17C and in *E. coli*. The results show that the *C. piscicola* clones produce mature BrcA and a fully functional truncated fragment of BrcB missing the first 9 amino acids, whereas the *E. coli* clones generate multiple fragments of BrcA or BrcB that are weakly active or inactive. Conformational studies by circular dichroism (CD) have been conducted on both the isolated bacteriocins and a mixture of the two purified peptides. In addition, an effective and easy purification method applicable to both BrcA and BrcB has been developed. A maltose-binding protein fusion of BrcA and an intein protein fusion of BrcB have also been constructed.

RESULTS AND DISCUSSION

1. Isolation of Various Bacteriocins Including Piscicolin 126 and Carnobacteriocins B1 and BM1 (CbnB1 and CbnBM1) from *C. piscicola* 307 and 682

C. piscicola 307 and 682 have recently been isolated by McMullen and co-workers (Department of Agricultural, Food and Nutritional Science, University of Alberta) from various meat products, and have been found to display activity against a broad range of Gram-positive bacteria including strains of *Listeria monocytogenes* and *Pediococcus*. In a collaborative effort to determine which bacteriocins are responsible for such antimicrobial activity, isolation, purification and genetic characterization of piscicolin 126, carnobacteriocins B1 and BM1, and of a new bacteriocin were achieved. The oligonucleotides used for primer extension in this study are listed in Table 5.

Table 5. Oligonucleotides utilized in this study

Primer	*Sequence*	*Description*
LG-126F1	5'-ATATGAATTCCGATGTTACAATCAATTAAC-3'	Complementary to forward piscicolin 126 nucleotide sequence (nucleotides 1-20)[305]
LG-126F2	5'-ATATGAATTCATGAAAACTGTTAAAGAACT-3'	Complementary to forward piscicolin 126 nucleotide sequence (nucleotides 136-156)[305]
LG-126R1	5'-ATATTCTAGACTTTTCCTCCAGAAAACCA-3'	Complementary to reverse 3' end of piscicolin 126 nucleotide sequence (nucleotides 600-582)[305]
SG BM1-F1	5'-ATATGAATTCATGAAAAGCGTTAAAGAACT-3'	Complementary to forward carnobacteriocin BM1 nucleotide sequence (nucleotides 102-122)[306]
SG BM1-R1	5'-ATATTCTAGATTAATGTCCCATTCCTGC-3'	Complementary to reverse nucleotide sequence of carnobacteriocin BM1 (nucleotides 288-270)[306]
SG BM1-R2	5'-ATATTCTAGATTAAAACCCTGACCAAGC-3'	Complementary to reverse nucleotide sequence of carnobacteriocin BM1 (nucleotides 569-552)[306]
SG B2-F1	5'-ATATTCTAGAATGAATAGCGTAAAAGAATTA-3'	Complementary to forward carnobacteriocin B2 nucleotide sequence (nucleotides 236-245)[306]
SG B2-R1	5'-ATAATCTAGATTACGGTCTCCTACCAAT-3'	Complementary to reverse nucleotide sequence of carnobacteriocin B2 (nucleotides 436-419)[306]
SG B2-R2	5'-ATAATCTAGATTAGAAATATATATAAGGACCG-3'	Complementary to reverse nucleotide sequence of carnobacteriocin B2 (nucleotides 809-790)[306]

1.1. Piscicolin 126

Piscicolin 126 is a well-characterized cystibiotic class IIa bacteriocin that was originally isolated from spoiled ham and characterized by Davidson and co-workers.[307] Cystibiotics are antibiotics containing at least one disulfide bridge essential for their activity.[308] Piscicolin 126 is heat-stable at low pH values (2-3), but loses all activity at neutral or alkaline pH values. Its spectrum of antimicrobial activity includes several Gram-positive bacteria (many strains of *Brochothrix thermosphacta, Carnobacterium, Enterococcus, Leuconostoc, Lactobacillus, Pseudomonas, Staphylococcus*, etc.), especially the food-borne pathogen *Listeria monocytogenes*, and spores of *Clostridium* and *Bacillus* species, but not Gram-negative bacteria or yeasts. This antimicrobial peptide contains 44 amino acid residues and has a molecular weight of 4416.6 ± 1.9 Da (Figure 13). Piscicolin 126 has found application as a starter culture in milk and Camembert cheese.[309]

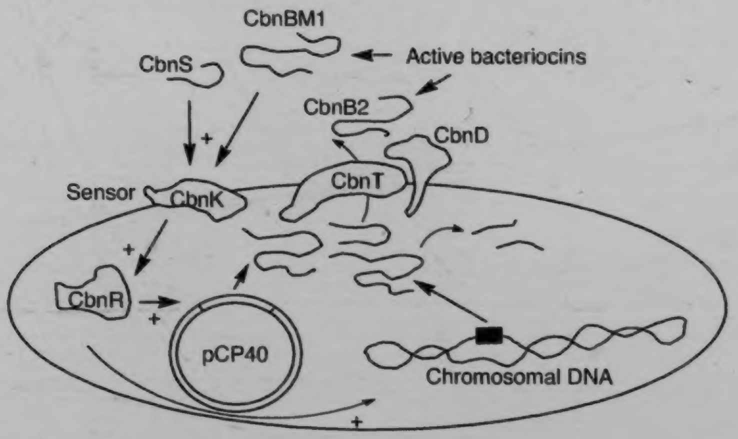
Figure 13. Single-strand DNA and amino acid sequences for nucleotides 1-600 of the *Hinf*I/*Sau*3A fragment containing the structural gene of piscicolin 126[305].

```
CGATGTTACAATCAATTAACTTTATAAGTTCATGAATAATATCGTGATAGTTCAGGAATA  60
AAAAATCTATAAGTAAAAAAGATGTGATACAGTCAGCATGTTGTAAAAAATATTTTAAAA 120

                 M  K  Y  V  K  E  L  S  V  K  E  M  Q  L  T
AGGAGCGTGTTTACGCATGAAAACTGTTAAAGAACTTAGCGTTAAAGAAATGCAACTAAC 180

 T  G  G  K  Y  Y  G  N  G  V  S  C  N  K  N  G  C  T  V  D
TACAGGAGGTAAGTATTACGGAAATGGCGTTTCCTGTAATAAAAATGGTTGTACTGTAGA 240

 W  S  K  A  I  G  I  I  G  N  N  A  A  A  N  L  T  T  G  G
TTGGAGCAAAGCTATTGGGATTATAGGAAACAATGCAGCAGCAAATTTGACTACAGGTGG 300

 A  A  G  W  N  K  G
AGCCGCTGGTTGGAACAAAGGATAATTAAAGTCTCTTATTTTTTATCTTGTAAAAAAGAT 360
GATACGCATCAATGCTGTGACATAACATAGATGGGTCTTTATATTTGTAAGTTACATTTA 420
AAACAAAAATAAATATATAAAAATATTTTTTTATAGTCTTAGGAATTATGTTATACTAAC 480
AAAAATAGGCTAGTTTCAACATGATGTAAAGAAACTTATACTATCAACTAAAATCATAAA 540
TATATAAAATTAAGGAGTGATATTTTATGGGTAAGTTAAAATGGTTTTCTGGAGGAAAAG 600
```

1.2. Bacteriocins of *Carnobacterium piscicola* LV17

The production of bacteriocins by *Carnobacterium piscicola* LV17 was first reported by Ahn and Stiles.[310] C. *piscicola* LV17B,[311] a *Lactobacillus*-type organism derivative of *C. piscicola* LV17 originally isolated from chill-stored meats,[306] produces three class IIa bacteriocins: carnobacteriocin A (CbnA), carnobacteriocin B2 (CbnB2), and carnobacteriocin BM1 (CbnBM1) (Figure 14). The production of CbnA, which is encoded on a separate plasmid is not shown in Figure 14. The chromosomal CbnBM1 production depends on the plasmid-mediated genetic information for CbnB2.[312]

Figure 14. Synthesis of CbnB2 and CbnBM1 by *C. piscicola* LV17[223,312]



pCP40 is a plasmid containing the genetic information for the production of CbnB2. The genes for CbnBM1 and immunity are represented by the box on the chromosome. pCP40 must be present for secretion of CbnBM1. CbnT, CbnD, CbnS, CbnK and CbnR are the ABC transporter, accessory protein, induction peptide, histidine kinase and response regulator, respectively.

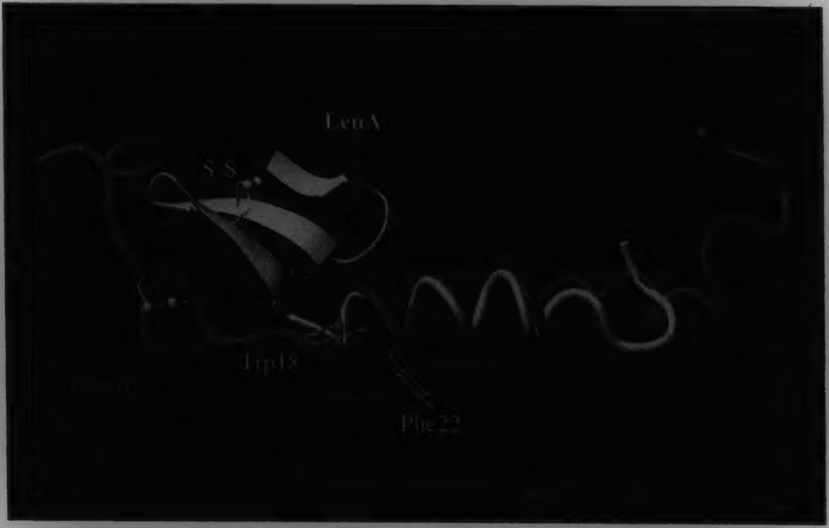
1.2.1. Carnobacteriocin B2 (CbnB2)

CbnB2 is a heat-stable, cationic bacteriocin that is active against many LAB and strains of potentially pathogenic *Listeria monocytogenes* and *Enterococcus* species.[306,313] Purification of this class IIa bacteriocin was achieved in 1994.[306] Genetic characterization of the *cbnB2* gene cluster present on plasmid pCP40 (61 kb) in *C. piscicola* LV17B revealed the presence of four ORFs.[312] The DNA and amino acid sequences for the structural gene *cbnB2* are depicted in Figure 15. An immunity gene, *cbiB2*, has been found downstream of *cbnB2* (structural gene).[274] Found upstream of *cbnB2* are several ORFs that produce proteins that are involved in regulation (*cbnR*), and that function as ABC transporter (*cbnT*), accessory protein (*cbnD*), induction peptide (*cbnS*), and histidine kinase (*cbnK*). Secretion of mature CbnB2 (48-amino acid peptide, 4569.9 ± 0.7 Da) occurs after posttranslational cleavage at the double-glycine site of the ribosomally synthesized prebacteriocin of CbnB2 (66 amino acid residues). The three-dimensional solution structure of CbnB2 has been elucidated and compared to the tertiary structure of LeuA (Figure 16).[304] It has been concluded that high sequence conservation of the N-terminal region does not generate an identical structure motif. It has also been postulated that the more variable sequences of the central region, which result in similar α-helices may be involved in receptor recognition.

Figure 15. Single-strand DNA and amino acid sequences for nucleotides 141-490 of the *Hind*III fragment from pLQ5.21 containing the structural gene of CbnB2. The *vertical arrow* in the amino acid sequence of the prebacteriocin indicates the cleavage site

```
AAATACCCTGGTTCAAGATGTATTTTCCAAAAAAATGTTCAGATATGATATAGTTTTTTT 200
                         -35                     -10
                                    M  N  S  V  K  E  L  N
GAAATACAAATATAAAATAAAGGAGTTTGATTTAGATGAATAGCGTAAAAGAATTAAACG 260
                     RBS     ↓
V  K  E  M  K  Q  L  H  G  G  V  N  Y  G  N  G  V  S  C  S
TGAAAGAAATGAAACAATTACACGGTGGAGTAAATTATGGTAATGGTGTTTCTTGCAGTA 320

K  T  K  C  S  V  N  W  G  Q  A  F  Q  E  R  Y  T  A  G  I
AAACAAAATGTTCAGTTAACTGGGGACAAGCCTTTCAAGAAAGATACACAGCTGGAATTA 380

N  S  F  V  S  G  V  A  S  G  A  G  S  I  G  R  R  P
ACTCATTTGTAAGTGGAGTCGCTTCTGGGGCAGGATCCATTGGTAGGAGACCGTAAATAT 440


ATAAATACAATATAGAGCAAGGTGGTGATACAATGGATATAAAGTCTCAA           490
                    RBS
```

Figure 16. Superimposition of ribbon diagrams of CbnB2 and LeuA based on the alignment of backbone atoms from Trp18 to Phe22[304]



1.2.2. Carnobacteriocins B1 and BM1 (CbnB1 and CbnBM1)

Carnobacteriocin BM1, produced by *C. piscicola* LV17B, belongs to the largest group of bacteriocins (class IIa).[312] It is a heat-stable cystibiotic that contains one disulfide bridge in the N-terminal half of the molecule. Its antibacterial spectrum includes *C. divergens*, *C. piscicola*, *L. plantarum*, *P. parvulus*, *E. faecium*, and strains of *Listeria* and *Enterococcus*. The nucleotide sequence encoding CbnBM1 has been previously established, and it is clear that this antimicrobial peptide (43-amino acid peptide, 4524.6 ± 0.6 Da) is the product of a prepeptide (61-amino acid peptide) containing a double-glycine cleavage site in the leader sequence.[306] Analysis of the nucleotide sequence revealed the presence of two ORFs: *cbnBM1* (structural gene), and *cbiBM1* (immunity gene) (Figure 17). The immunity gene, located downstream of *cbnBM1*, encodes for an 88-amino acid peptide. Carnobacteriocin B1 (43-amino acid peptide, 4541.9 ± 0.6 Da) is the oxidized form of CbnBM1, which results from oxidation of the sulfur of the Met-41 residue of CbnBM1.

Many of the bacteriocins that belong to class IIa are characterized by a high degree of sequence homology in the N-terminal amino acid residues, whereas the C-terminal amino acids vary. This is reflected when comparing CbnB2 and CbnBM1, which show 72% identity between the leader regions, and 34% between the mature antimicrobial peptides (Figure 18).

Figure 17. Single-strand DNA and amino acid sequences for nucleotides 0-675 of the sequenced fragment containing the structural (*cbnBM1*) and ORF-α2 genes (*cbiBM1*) of carnobacteriocin BM1. The *vertical arrow* in the amino acid sequence of the prebacteriocin indicates the cleavage site

```
TCGAGATACGTTTATCCATGGTTCAGGATGATTTTATCAACGAGTTAATTATTTATGCTACAGTAAACTTGTTACTAAATACTTTATAAGGAGTGTATGT  100
                                                                                         RBS
                                                                         M  K  S  V  K  E  L  N  K
ACATGAAAAGCGTTAAAGAACTAAATAAAAAAGAAATGCAACAAATTAATGGTGGAGCTATCTCTTATGGCAATGGTGTTTATTGTAACAAAGAGAAATG  200
                                                                    cbnBM1→
                           ↓
K  E  M  Q  Q  I  N  G  G  A  I  S  Y  G  N  G  V  Y  C  N  K  E  K  C  W  V  N  K  A  E  N  K  Q  A
TTGGGTAAACAAGGCAGAAAACAAACAAGCTATTACTGGAATAGTTATCGGTGGATGGGCTTCTAGTTTAGCAGGAATGGGACATTAAAGAGGTATCTAG  300
                                                                                          RBS
  I  T  G  I  V  I  G  G  W  A  S  S  L  A  G  M  G  H                    M  I  K  D  E  K  I  N  K
TTATGATAAAAGATGAAAAAATAAATAAAATCTATGCTTTAGTTAAGAGCGCACTTGATAATACGGATGTTAAGAATGATAAAAAACTTTCTTTACTTCT  400
                                                                    cbiBM1→
I  Y  A  L  V  K  S  A  L  D  N  T  D  V  K  N  D  K  K  L  S  L  L  L  M  R  I  Q  E  T  S  I  N  G
TATGAGAATACAAGAAACATCAATTAATGGAGAACTATTTTACGATTATAAAAAAGAATTACAGCCAGCTATTAGTATGTACTCTATTCAACATAACTTT  500

  E  L  F  Y  D  Y  K  K  E  L  Q  P  A  I  S  M  Y  S  I  Q  H  N  F  R  V  P  D  D  L  V  K  L  L
CGGGTTCCTGACGATCTAGTAAAACTGTTAGCATTAGTTCAAACACCTAAAGCTTGGTCAGGGTTTTAACTTTAGTTCCAGATGAGTTAAAATCCTTAAA  600

 A  L  V  Q  T  P  K  A  W  S  G  F
AATAAGGAATAATGGTAAATCAGCATTCCTTATTTTTATAGTCATCACACTATAACTTTACTTAAAGATGTTCGA                            675
```

Figure 18. Alignment of the double-glycine-type leader peptides of the bacteriocin precursors CbnB2 and CbnBM1 and amino acid sequences of the mature bacteriocins

```
Leader of CbnB2:   MNSV-KELNVKEMKQLHGG-
Leader of CbnBM1:  MKSV-KELNKKEMQQINGG-

Mature CbnB2:        VNYGNGVSCSKTKCSVNWGQAFQERYTAGINSFVSGVASGAGSIGRRP
Mature CbnBM1:      AISYGNGVYCNKEKCWVNKAENKQAITGIVIGGWASSLAGMGH
```

1.3. Purification and Characterization of Piscicolin 126, CbnB1, and CbnBM1 from *C. piscicola* 307 and 682, and of a New Bacteriocin from *C. piscicola* 307

1.3.1. Purification of Bacteriocins from *C. piscicola* 307 and 682

C. piscicola 307 and 682 have been previously isolated, but the bacteriocins that they produce had not been purified and characterized. To achieve isolation of bacteriocins from these strains of carnobacteria, a novel 4-step purification method was developed. The procedure consists of a series of four different chromatography steps: an Amberlite XAD-16 resin column, a reversed-phase Varian MEGA BE-C_{18} column, a size exclusion Sephadex G25 column, and a reversed-phase C_8 HPLC column. To avoid loss of antimicrobial activity, all fractions collected during HPLC experiments must be kept on dry ice under argon. Alternative purification methods, including *n*-butanol and chloroform extraction followed by acetonitrile precipitation, which is useful for the purification of brochocin C and its components, did not allow for recovery of the desired active peptides. The antimicrobial activity results observed for each organism after the three first stages of the developed purification method are summarized in Table 6. Even though there is a loss in activity during the size exclusion column, it is a key step as it allows for the removal of several small inactive peptides that would render the final HPLC purification impossible.

Table 6. Purification of APT cultures of *C. piscicola* 307 and 682

Organism	*Purification stage*	*Vol (mL)*	*Activity*[a] *(AU/mL)*	*Total activity (AU)*	*Recovery (%)*
C. piscicola 307	Culture supernatant	1,000	800	8.0×10^5	100
	Amberlite XAD-16 (leave in H_2O)	25	25,600	6.4×10^5	80
	Varian MEGA BE-C_{18} (leave in H_2O)	2	102,400	2.0×10^5	25
	Sephadex G25 (leave in 20% IPA)	1.5	12,800	1.9×10^4	2.4
C. piscicola 682	Culture supernatant	1,000	800	8.0×10^5	100
	Amberlite XAD-16 (leave in H_2O)	25	12,800	3.2×10^5	40
	Varian MEGA BE-C_{18} (leave in H_2O)	2	102,400	2.0×10^5	25
	Sephadex G25 (leave in 20% IPA)	1.5	51,200	7.7×10^4	10

[a] Activity tested against *C. divergens* LV13.

Four peptides active against *C. divergens* LV13 were isolated from *C. piscicola* 307 by reversed-phase HPLC. When subjected to the same HPLC method, only two active peptides were recovered from *C. piscicola* 682. All these antimicrobial compounds were analyzed by MALDI-TOF mass spectrometry and amino acid sequencing.

1.3.2. Mass Spectrometry and Amino Acid Sequence

All the isolated peptides could be detected by MALDI-TOF mass spectrometry using sinapinic acid as a matrix. The mass results obtained for bacteriocins of *C. piscicola* 307 and 682 from HPLC purification are reported in Table 7 and depicted in Figure 19. The molecular masses of 4414.7, 4523.8, and 4540.6 Da seemed to indicate the presence of piscicolin 126, CbnBM1, and CbnB1, for which molecular masses of 4416.6 ± 1.9, 4524.6 ± 0.6, and 4541.9 ± 0.6 Da have been reported, respectively. The molecular mass of 5863.5 could not be correlated to any known bacteriocin.

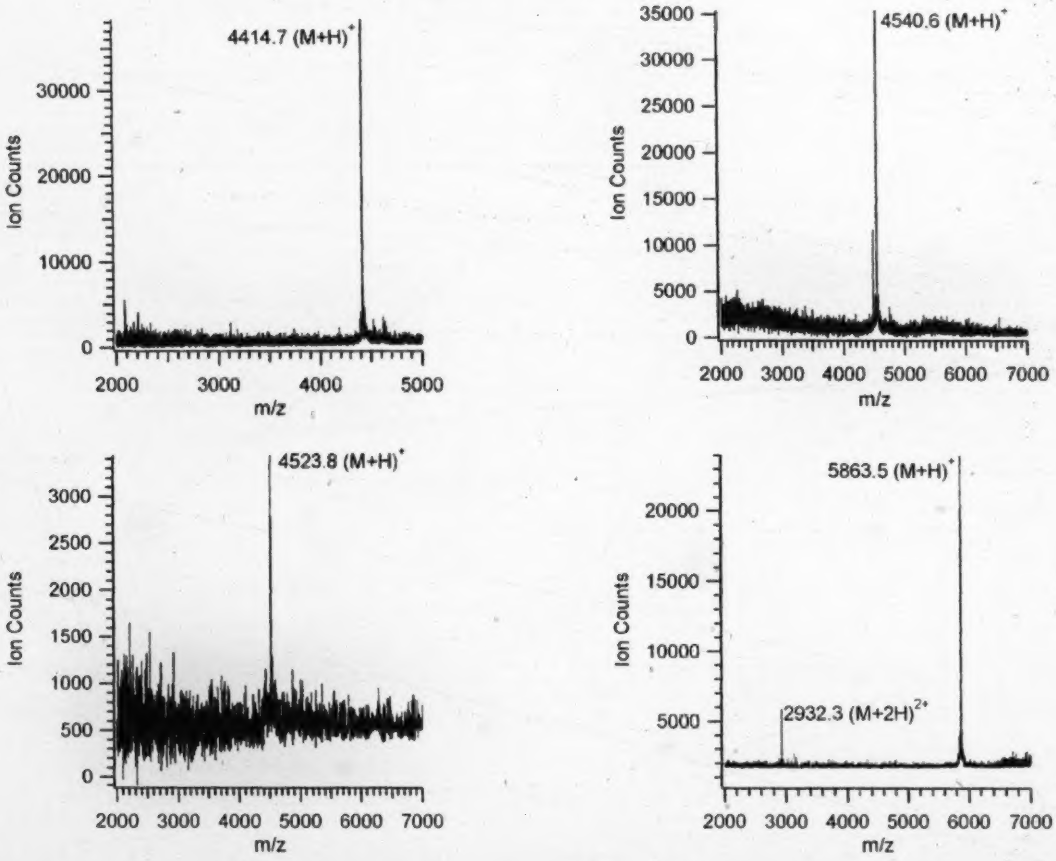
Table 7. MALDI-TOF MS of antimicrobial compounds of *C. piscicola* 307 and 682[a]

Organism	*Retention time on HPLC (min)*	*MW (Da)*	*Bacteriocin*
C. piscicola 307	13.1-14.7	4523.8	CbnBM1
		4540.6	CbnB1
	14.0-16.4	4414.7	Piscicolin 126
		4523.8	CbnBM1
	45.2-47.9	5863.5	Unknown
C. piscicola 682	14.8-15.8	4414.7	Piscicolin 126
		4523.8	CbnBM1
	15.5-16.9	4414.7	Piscicolin 126

[a] Data obtained after reversed-phase C_8 HPLC.

To determine or confirm the identity of these bacteriocins, N-terminal amino acid sequencing analyses were performed on the pure HPLC samples. Unfortunately, no N-terminal amino acid sequence could be obtained for the unknown bacteriocin, which appears blocked to Edman sequencing at the N-terminus. The amino acid sequence analysis of piscicolin 126 identified the first 25 residues of the desired peptide: NH_2-KYYGNGVSCNKNGCTVDWSKAIGII. The cysteines (underlined in the sequence) could not be detected as they are part of a disulfide bridge. The amino acid sequence analysis of CbnB1 identified the first 33 amino acid residues and residue 35 of the oxidized peptide (excluding the two cysteines of a disulfide bridge): NH_2-AISYGNGVYCNKEKCWVNKAENKQAITGIVIGG-A-. For an unknown reason no N-terminal amino acid sequence could be obtained for samples of CbnBM1. However, the identity of CbnBM1 was confirmed by the amino acid sequence of CbnB1, a form of CbnBM1 wherein the methionine at position 41 is oxidized to a sulfoxide.

Figure 19. Mass spectra obtained for piscicolin 126, CbnB1, CbnBM1, and an unknown antimicrobial compound after purification of 1-liter fermentation cultures of *C. piscicola* 307 and 682



1.3.3. Genetic Characterization of Piscicolin 126 and CbnBM1 from *C. piscicola* 307 and 682

In addition to the results obtained by mass spectrometry and amino acid sequencing, the presence of the genetic determinants for piscicolin 126 in *C. piscicola* 307 and 682 was verified by analysis of part of the genetic sequence found in these strains of carnobacteria. PCR experiments on *C. piscicola* 307 and 682 using combinations of primers LG-126 F1 and LG-126R1, and LG-126F2 and LG-126R1, agarose gel electrophoresis, and DNA sequencing experiments confirmed the presence of the structural gene encoding piscicolin 126 in both strains.

1.3.4. Determination of Possible Production of CbnB2 by *C. piscicola* 307 and 682

In *Carnobacterium piscicola* LV17 it has been established that activation and export from the cell of CbnBM1, which has its structural gene located on the chromosome, depend on the plasmid-mediated genetic information for carnobacteriocin B2[312] (Figure 14). Even though the presence of CbnB2 in *C. piscicola* 307 and 682 could not be detected at any stage of the developed purification method, its production by *C. piscicola* 307 and 682 was suspected as these bacteria produce CbnBM1. Genetic manipulations were performed to determine if the gene encoding CbnB2 is indeed present in *C. piscicola* 307 and 682. Preliminary studies including PCR experiments on *C. piscicola* 307 and 682 using combinations of primers SG B2-F1 and SG B2-R1, and SG B2-F1 and SG B2-R2, agarose gel electrophoresis, and DNA sequencing experiments confirmed the absence of the gene encoding CbnB2 in *C. piscicola* 307 and 682. These results are of great interest as they suggest that production of CbnBM1 in *C. piscicola*

307 and 682 does not require the complementing *trans*-acting factor encoded in the genetic information for CbnB2 as previously reported by Quadri.[312] More details on the genetic organization in these two new strains of carnobacteria are required to understand this new mode of production of CbnBM1.

1.3.5. Antimicrobial Spectrum of Activity of Piscicolin 126, CbnBM1 and Unknown Bacteriocin from *C. piscicola* 307 and 682

Preliminary studies by Stiles and co-workers on the antibacterial activity spectrum of *C. piscicola* 307 and 682 revealed that *C. piscicola* 307 is more potent than 682. In an effort to understand what bacteriocin is responsible for the greater activity of *C. piscicola* 307, all isolated pure and mixed peptides from strains 307 and 682 were tested for antimicrobial activity against various strains of *Listeria*, *C. divergens*, and *Pediococcus* species. The inhibitory activity results obtained are reported in Table 8.

Table 8. Antimicrobial spectrum of activity of *C. piscicola* 307 and 682[a]

Organism	*Peptides*	*NTCC 5105.3*[b] *(AU)*	*HPB 642*[b] *(AU)*	*CDC 7762*[b] *(AU)*	*FS15*[b] *(AU)*	*15313*[b] *(AU)*	*LV13*[c] *(AU)*	*PAC 1.0*[d] *(AU)*	*Pronase E (AU)*
307	Control[e]	6400	1600	6400	6400	1600f[f]	400	100	+[g]
307	CbnBM1	400	100f	400	400	400f	100	0	+
307	CbnBM1 and piscicolin 126	>6400	>6400	3600	>6400	6400	1600	0	+
307	Unknown[h]	400	400	200vf	400	200	800	1600	+
682	Control	>6400	>6400	1600	>6400	3200	400	0	+
682	piscicolin 126	1600	3200	400	6400	800	200	0	+

[a] Samples partially and totally purified by HPLC. [b] *Listeria* species. [c] *C. divergens* species. [d] *Pediococcus* species. [e] Supernatant after growth of the organism. [f] f means faint and vf means very faint. [g] Activity of the bacteriocin inhibited by pronase E. [h] Pure unknown peptide.

These data suggest that the activity observed against *Pediococcus* species in control studies with *C. piscicola* 307 results from the expression of the new unknown bacteriocin isolated in this specific strain of carnobacteria. Studies are currently underway to determine the amino acid sequence of this new antimicrobial compound.

2. Studies on BrcA and BrcB, Components of the Two-Peptides Bacteriocin BrcC

2.1. Primary Structure, Production, Regulation of Brochocin C

Brochothrix campestris ATCC 43754 produces a heat-stable, two-component nonlantibiotic class IIb bacteriocin, brochocin C (BrcC), that was originally discovered by Siragusa and Nettles Cutter,[314] and partially purified and characterized by McCormick.[277] Genetic analysis of the BrcC operon revealed the presence of five ORFs: *brcA* and *brcB* (structural gene), *brcI* (immunity gene), *brcT* and *brcD* (Figure 20). It is believed that the *brcI* gene, found downstream and overlapping the *brcB* gene, encodes for a 53-amino acid peptide. Also found downstream of the *brcI* gene are *brcT* and *brcD* that encode proteins with homology to ATP-binding cassette translocator and accessory proteins. In *Brochothrix campestris*, mature BrcA (59-amino acid peptide, 5245 Da) and BrcB (43-amino acid peptide, 3945 Da) result from the cleavage of the N-terminal leader peptide of the inactive prepeptide following the double-glycine cleavage sites. Fusion of the nucleotides encoding a divergicin A signal peptide in front of the *brcA* and *brcB* structural genes in *Carnobacterium piscicola* allowed independent expression of each component by the general *sec*-pathway.[277]

Figure 20. Single-strand DNA and amino acid sequences for nucleotides 1201-1900 of the *Eco*RI fragment of pAP7.4 containing the structural (*brcA* and *brcB*) and immunity (*brcI*) genes of brochocin C

```
                                                                         M  H  K  V  K  K  L  N  N
TTAACCAATAGTAAAAATTATCATGCTATCTTTTGTATGTAATAAAAATTATTTAAAGGAGGGTGTTTCATCATGCACAAGGTAAAAAAATTAAACAATC 1300
            -10                                         RBS           brcA→
Q  E  L  Q  Q  I  V  G  G  Y  S  S  K  D  C  L  K  D  I  G  K  G  I  G  A  G  T  V  A  G  A  A  G  G
AAGAGTTACAACAGATCGTGGGAGGTTACAGTTCAAAAGATTGTCTAAAAGATATTGGTAAAGGAATTGGTGCTGGTACAGTAGCTGGGGCAGCCGGCGG 1400

  G  L  A  A  G  L  G  A  I  P  G  A  F  V  G  A  H  F  G  V  I  G  G  S  A  A  C  I  G  G  L  L  G
TGGCCTAGCTGCAGGATTAGGTGCTATCCCAGGAGCATTCGTTGGAGCACATTTTGGAGTAATCGGCGGATCTGCCGCATGCATTGGTGGATTATTAGGT 1500

 N                 M  K  K  E  L  L  N  K  N  E  M  S  R  I  I  G  G  K  I  N  W  G  N  V  G  G  S
AACTAGGAGGTTATATTTATGAAAAAAGAACTATTGAATAAAAATGAAATGAGTAGAATTATCGGCGGCAAAATAAATTGGGGAAATGTTGGCGGTTCTT 1600
     RBS         brcB→
                                                                                               M  V
C  V  G  G  A  V  I  G  G  A  L  G  G  L  G  G  A  G  G  G  C  I  T  G  A  I  G  S  I  W  D  Q  W
GTGTTGGAGGTGCAGTAATTGGAGGCGCCCTCGGTGGACTAGGTGGAGCTGGCGGAGGTTGCATTACAGGAGCTATCGGAAGTATTTGGGATCAATGGTA 1700
                                                                              RBS       brcI→
 K  T  I  L  F  S  V  V  I  S  F  V  A  L  C  N  F  L  I  K  K  D  V  S  S  K  K  K  L  F  L  T  G
AAAACTATACTATTTTCGGTTGTAATTTCATTCGTTGCATTATGTAACTTTTTAATAAAAAAAGATGTGTCTTCAAAAAAAAAATTATTTTTAACAGGTT 1800

S  I  A  V  F  L  I  I  Y  D  F  L  W  I  I  F  S  N
CTATTGCTGTCTTTCTAATTATCTATGATTTTCTATGGATTATATTCTCTAACTAGTACAAAGCTATATCTATATGTGTTAAATAATTATATAACAATAA 1900
```

2.2. Antibacterial Spectrum of BrcC

The cytoplasmic membrane of Gram-positive bacteria is the primary target for the action of bacteriocins. The outer membrane of Gram-negative bacteria, impenetrable to these antibiotic peptides, usually prevents antimicrobial activity. Brochocin C has a broad activity spectrum comparable to that of nisin. The inhibition spectrum includes a broad range of Gram-positive bacteria (35 strains of *B. thermosphacta*, 10 strains of *Listeria monocytogenes*, many strains of *Carnobacterium*, *Enterococcus*, *Kurhtia*, *Lactobacillus*, and *Pediococcus*) and spores of *Clostridium* and *Bacillus* species, but not Gram-negative bacteria (29 strains tested).[277,315] Stiles has recently confirmed that the outer membrane of a Gram-negative bacterium protects the cytoplasmic membrane by acting as a barrier towards BrcC.[316] Experiments involving addition of BrcC to energized cells and spheroplasts of *Salmonella typhimurium* resulted in hydrolysis of ATP and showed that this bacteriocin affects Gram-negative bacteria when their outer membranes are impaired. Related studies on other two-peptide bacteriocins, lactacin F[317] and thermophilin 13,[318] have led to similar conclusions that formation of poration complexes occurs in the cytoplasmic membrane of Gram-negative bacteria. The results suggest that the fluidity of the membrane may influence the action of these antimicrobial peptides.

2.3. Plasmids and Producer Strains Utilized in this Study

The bacterial strains and plasmids used in this study are listed in Table 9, except for the target bacteria used to determine the antibacterial spectrum of BrcC.

Table 9. Plasmids and producer strains utilized in this study

Plasmid and producer strain	***Relevant properties***[a]	***Ref. or source***
Plasmids		
pT712	Amp^r, 2.8 kb, T7-polymerase expression vector	319
pJKM46	pRW19e containing 309-bp *Hind*III-*Kpn*I, *dvn::brcB brcI* fragment, 4.0 kb, Em^r	277
pJKM61	292-bp *Xba*I-*Sac*I fragment from pJKM53-1 and 426-bp *Sac*I-*Kpn*I fragment from pJKM46 cloned in *Xba*I and *Kpn*I sites of pUC118, *dvn::brcA dvn::brcB brcI*, Amp^r, 3.9 kb	277
pJKM56	292-bp *Xba*I-*Sac*I fragment from pJKM53-1 and 444-bp *Sac*I-*Kpn*I background chromosomal fragment cloned in *Xba*I and *Kpn*I sites of pMG36e, *dvn::brcA*, Em^r, 4.3 kb	277
pMG36e	3.6 kb, Em^r 6	320
pSG1	292-bp *Xba*I-*Sac*I fragment from pJKM61 cloned in *Xba*I and *Sal*I sites of pT712, *dvn::brcA*, Amp^r, 3.1 kb	This study
pSG15	PCR fragment from pJKM46 cloned in *Xba*I and *Eco*RI sites of pT712, *dvn::brcB*, Amp^r	This study
pMAL™-p2X	Amp^r, 6721-bp, *lacI*, *lacZα* and *malE* expression vector	321-324
pSG618422	PCR fragment from pJKM56 cloned in *Sal*I site of pMAL™-p2X, Amp^r	This study
pSG619685	PCR fragment from pJKM46 fused to intein fragment of pTYB11 cloned in *Xba*I and *Sal*I sites of pTYB11, Amp^r	This study
pTYB11	Amp^r, 7412-bp, *lacI*, T7-polymerase expression vector	325,326
Strains		
E. coli BL21(DE3)	F *ompTr$_B$m$_B$int*; bacteriophage DE3 lysogen carrying the T7 RNA polymerase gene controlled by the *lac*UV5 promoter	327
E. coli ER2566	Fλ *fhuA2 [lon] ompT lacZ::T7 gene1 gal sulA11 Δ(mcrC-mrr0114::IS10 R(mcr-73::miniTn10-TetS02 R(zgb-210::Tn10)(TetS) endA1 [dcm]*	NE BioLabs
E. coli TB1	F *araΔ(lac-proAB)[φ80dlacΔ(lacZ)M15]rpsL(Str^R) thi hsdR*	NE BioLabs
C. piscicola UAL26	Plasmid free, produces uncharacterized bacteriocin, brochocin C sensitive	Lab. collection
C. divergens LV13	Wild-type divergicin A producer, brochocin C sensitive	276
C. piscicola LV17C	Plasmid free, brochocin C sensitive	313

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[a] Amp^r, ampicillin resistant; Em^r, erythromycin resistant; *dvn::brcA*, brochocin C peptide A gene fused to DNA encoding divergicin A signal peptide; *dvn::brcB*, brochocin C peptide B gene fused to DNA encoding divergicin A signal peptide; *brcI*, brochocin C immunity gene.

2.4. Purification and Characterization of BrcA and BrcB(10-43), a Functional Fragment Generated by Heterologous Expression in *C. piscicola*

2.4.1. Purification of BrcA and BrcB from Carnobacteria

Separate expression clones of BrcA (pJKM56) and BrcB (pJKM46) have been previously constructed in *Carnobacterium piscicola* LV17C, but the products have not been chemically characterized. The purification procedure previously described for BrcC by McCormick *et al.*[277] did not allow successful isolation of both components from 3-liter fermentation cultures of pJKM56 and pJKM46 in a modified semidefined CAA medium. Their loss at the Sephadex G50 column chromatography step is probably due to very strong binding to the matrix. BrcA and BrcB are quite intractable and bind either irreversibly or with degradative activity loss to a variety of solids, including silica gel (both normal and C_{18}, C_8, and C_4 reversed-phase), polystyrene, cellulose and various HPLC column supports. A new 3-step purification method consisting of *n*-butanol and chloroform extraction, and size exclusion chromatography was developed. Acetonitrile precipitation, useful for BrcC purification, did not prove to be necessary for obtaining pure compounds. It is known that Tween 80, a detergent usually used to prevent bacteriocins from sticking to the bacterial cells and the glassware, stops the adsorption of nisin and enterocin on polypropylene surfaces.[328] As Tween 80 interferes with purification of the desired peptides and hampers mass spectral analyses, it was eliminated from the original CAA medium.[329] As both pure peptides precipitate in aqueous solution (> 8% H_2O), Sephadex LH-20, which permits the use of organic solvents, was chosen as the solid support for size exclusion chromatography. Attempts at purifying BrcA by HPLC using chloroform and methanol were unsuccessful. The antimicrobial activity results observed at each stage of the developed purification method are summarized in Table 10. Using this new purification procedure, about 2 mg of pure peptide could be recovered per liter of fermentation culture. The final specific activities of purified BrcA and BrcB fractions were 706000 and 800 AU/mg respectively. As most of the antimicrobial activity is lost during Sephadex LH-20 size exclusion chromatography, this step was eliminated from the purification procedure. As expected, the BrcA peptide only displayed antimicrobial activity against the indicator strain LV17C(pMG36e) in the presence of BrcB or the BrcB-producing organism, whereas the BrcB peptide required BrcA or its producing organism for activity.

Table 10. Purification of modified CAA cultures of BrcA (pJKM56) and BrcB (pJKM46)

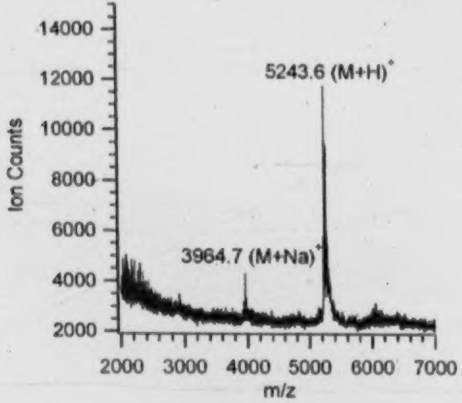
Plasmid	*Purification stage*	*Vol (mL)*	*Activity*[a] *(AU/mL)*	*Total activity (AU)*	*Activity recovered (%)*
pJKM56	Culture supernatant	1,000	3,200	3.2×10^6	100
	n-BuOH extraction (leave in H_2O)	10	204,800	2.0×10^6	63
	$CHCl_3$ extraction	1	819,200	8.2×10^5	26
pJKM46	Culture supernatant	3,000	12,800	3.8×10^7	100
	n-BuOH extraction (leave in H_2O)	40	409,600	1.6×10^7	42
	$CHCl_3$ extraction	4	51,200	2.0×10^5	0.53

[a] Activity tested against LV17C(pMG36e).

Despite the loss of some peptide during chloroform extraction, it is a key step for purification of both of these very hydrophobic compounds because it allows removal of most hydrophilic contaminants. The new purification procedure can also be applied effectively up through the chloroform extraction step to the BrcC complex produced by *B. campestris*. Mass spectral analysis of the two components of BrcC from the parent *B.*

campestris producer demonstrates that the mature peptides which are expected on the basis of genetic analysis[277] are in fact generated (Figure 21).

Figure 21. Mass spectrum obtained for BrcC after $CHCl_3$ extraction



2.4.2. Activity and Stability of BrcA and BrcB

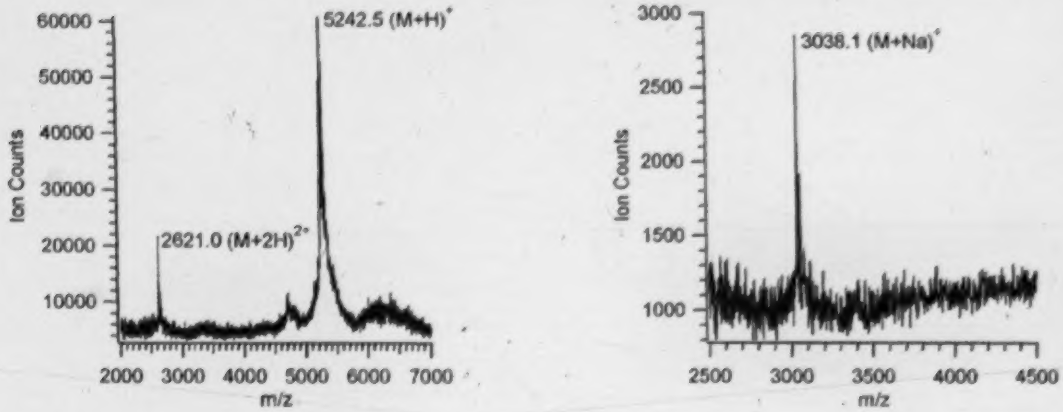
Samples of both peptides from carnobacteria were tested for stability under different storage conditions (temperature: -20, 4, and 25 °C; under argon or exposed to the air) after each step of the developed purification procedure. Residual activity was measured by the spot-on-lawn assay against LV17C(pMG36e) every 24 h for 4-6 days depending on the samples. Although BrcC is stable from pH 2 to 9 at 100 °C,[277,314] the BrcA and BrcB components were found to be much less robust. The degree of purity of the sample was also found to have an effect on its stability. Although very crude extracts may be more prone to degradation, for somewhat purer samples, other contaminating peptide impurities seem to protect BrcA and BrcB from decomposition. Pure samples of BrcA and BrcB exposed to the air were found to lose all bactericidal activity in less than 48 h, whereas pure peptides kept under argon stayed active for months when stored at -20

°C. Based on the results obtained, the best storage conditions were determined to be at or below -20 °C under an inert atmosphere of argon.

2.4.3. Mass Spectrometry and Amino Acid Sequence

Both components of BrcC produced by *B. campestris* could be detected by MALDI-TOF mass spectrometry using α-cyano-4-hydroxycinnamic acid as a matrix, after the chloroform extraction step. No other matrices were successful for detection of the desired peptides. Methods to detect bacteriocins by MALDI-TOF mass spectrometry in highly contaminated culture supernatants have recently been reported.[330] However, BrcB is difficult to detect by mass spectrometry until a reasonable state of purity is attained. The molecular masses of BrcA and BrcB were 5244 and 3944, respectively, in good agreement with the masses predicted from the genetic sequence,[277] which are 5245 and 3945, respectively. Analogous purification of BrcA from expression clone pJKM56 in *C. piscicola* LV17C using the divergicin A leader[225] gave pure peptide whose mass of 5242.5 Da indicates that the desired mature peptide was isolated (Figure 22). However, the molecular weight of 3038.1 Da observed for the active peptide isolated from the BrcB expression clone pJKM46 in *C. piscicola* LV17C indicates that the expected mature peptide has been cut after the double glycine found at positions 8 and 9, and that the isolated compound is a fragment consisting of amino acids 10-43, designated as BrcB(10-43) (Figure 22).

Figure 22. Mass spectra obtained for pure BrcA and pure BrcB(10-43) after purification of 3-liter fermentation cultures of pJKM56 and pJKM46, respectively



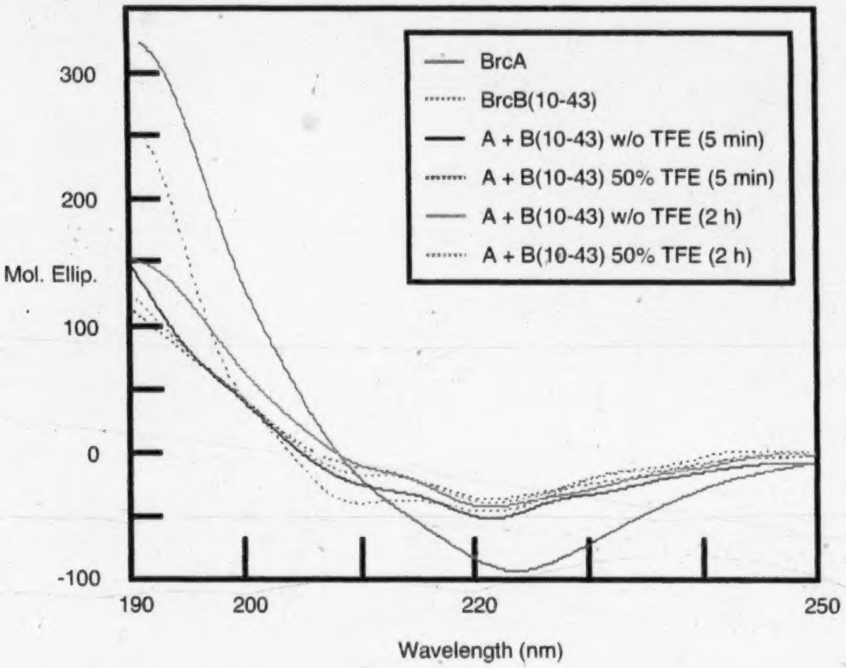
Presumably, the bacteriocin production machinery present in *C. piscicola*[312] recognizes the expressed precursor bearing the leader as a prebacteriocin to be processed at the common Gly-Gly site. This cleavage is likely accomplished by an ABC transporter having cysteine proteinase activity.[275,331] It seems unlikely that the peptide is first exported by the *sec*-pathway (mediated by the leader)[234,276] and then processed by the bacteriocin transporter system, but this possibility cannot be rigorously excluded at present. BrcB(10-43) displays the expected requirement from complementation by BrcA for antimicrobial activity. Interestingly, the BrcB(10-43) shows all of the properties expected for the complete BrcB peptide. Most importantly, it is inactive by itself, but complements BrcA to give a potent antimicrobial complex whose activity level and spectrum cannot be distinguished from that of native BrcC.

The amino acid sequence analysis of BrcA after chloroform extraction identified the first 31 residues of the desired peptide excluding the starting Y: NH_2-SSKDCLKDIGKGIGAGTVAGAAGGGLAAGL. No N-terminal amino acid sequence of BrcB(10-43) could be obtained due to the hydrophobicity of this peptide and solubility problems encountered when trying to perform sequencing.

2.4.4. Conformational Studies by Circular Dichroism

Thus far, circular dichroism (CD) studies[332] and NMR solution structures[303,304] of type II nonlantibiotic bacteriocins having 48 amino acids or fewer indicate that they usually have random coil conformations in pure water and assume defined three-dimensional structures only upon addition of lipid micelles or trifluoroethanol (TFE). Such experiments are not feasible with BrcA and BrcB(10-43) because of their very low water solubility. However, in order to investigate the secondary structure of both purified peptides, BrcA and BrcB(10-43), individually and as a mixture, circular dichroism measurements were taken in methanol with and without TFE. It was observed that both peptides were well structured in methanol and that the presence of TFE did not induce any changes in their degree of secondary structure. The high content of β-sheets found in both BrcA and BrcB(10-43), and in a 1:1 mixture of the two peptides (Figure 23) may in part account for their self-association and intractable properties.

Figure 23. CD spectra of BrcA and BrcB(10-43), individually and as a 1:1 mixture in MeOH with and without TFE



2.5. Studies on Growth Conditions for Isotopic Labeling of BrcA

A desirable objective of such studies is to elucidate the high-resolution tertiary structure of both components of BrcC to obtain insights into the mechanism of action of this protein. With the new purification method developed for the isolation of BrcA and BrcB(10-43), various growth conditions were explored to achieve isotopic labeling of these peptides for future NMR studies. Reports by Wang[333] and Wood[334] on fermentation methods that allow ^{13}C and ^{15}N labeling of proteins in *Pichia pastoris* have recently been published. The novel ^{15}N *Pichia pastoris* GS115 medium,[335] which successfully yielded ^{15}N labeled scFvB80, seemed to be a good starting point for our growth condition studies. Unfortunately, attempts at growing unlabeled *P. pastoris* GS115 did not furnish sufficient quantity of yeast to prepare the large amount of media required for the production of BrcA or BrcB. The method was abandoned and our attention was focused on the use of a procedure for universal ^{13}C and/or ^{15}N labeling of bacteriocins developed in our group.[336]

2.5.1. Growth of BrcA and BrcB in *C. piscicola* Using Complex Peptone Medium[336]

Using peptone from cyanobacterium *Anabaena* sp. ATCC 27899 as a source of ^{13}C and ^{15}N previously allowed our group to isotopically enrich nisin A and leucocin A,[336] and recently permitted labeling of subtilosin A.[337] The method utilizes $NaH^{13}CO_3$ and $Na^{15}NO_3$ to produce ~5 g of ^{13}C and ^{15}N labeled hydrolysate per 8 L culture of blue-green algae. Preliminary studies using unlabeled peptone (1 g/100 mL of medium) as a source of carbon and nitrogen to grow pJKM56 and pJKM46 show that both BrcA and BrcB(10-43) respectively, are produced in this medium. Identification of both peptides was done by mass spectrometry and activity assay. The antimicrobial activity results observed at each stage of our newly developed purification method (Section 2.4.1) are summarized in Table 11. Using this complex medium, the products that were obtained displayed good antimicrobial activity. However, they were much less clean than when the modified CAA medium was employed. The purification problems encountered after the chloroform extraction as well as the time and expenses involved in the preparation of this complex medium persuaded us to look for alternative growth conditions to produce the desired labeled peptides.

Table 11. Purification of peptone[336] cultures of BrcA (pJKM56) and BrcB (pJKM46)

Plasmid	*Purification stage*	*Vol (mL)*	*Activity[a] (AU/mL)*	*Total activity (AU)*	*Activity recovered (%)*
pJKM56	Culture supernatant	500	1,600	8.0×10^5	100
	n-BuOH extraction (leave in H_2O)	10	409,600	4.1×10^6	100
	$CHCl_3$ extraction	2	16,384	3.3×10^6	80
pJKM46	Culture supernatant	400	1,600	6.4×10^5	100
	n-BuOH extraction (leave in H_2O)	10	51,200	5.1×10^5	80

[a] Activity tested against LV17C(pMG36e).

2.5.2. Purification of BrcA in *C. piscicola* Using Celtone®-U Complete Medium

In an attempt to reduce the amount of time involved in the preparation of the labeled growth medium, preliminary studies using the commercially available unlabeled Celtone®-U complete medium (5013L0) (Spectra Stable Isotopes, Columbia, MD, USA) were performed on BrcA. Using the previously developed purification method (Section 2.4.1) pure BrcA could be obtained. Identification of the desired peptide was achieved by mass spectroscopy analysis and activity assay. The results of antimicrobial activity measured at each stage of the purification process are summarized in Table 12. Comparison of the results of total activity obtained with this medium and the modified CAA medium (Table 10) reveal that the Celtone®-U complete medium does not produce as much of the desired BrcA. The loss of peptide during the chloroform extraction is also more noticeable when the Celtone®-U medium is utilized, only 0.4% recovery compared to 26% when the modified CAA medium is used. These growth conditions were abandoned and the use of a defined labeled medium was envisaged as an alternative route for the production of labeled BrcA.

Table 12. Purification of a Celtone®-U complete medium culture of BrcA (pJKM56)

Purification stage	*Vol (mL)*	*Activity*[a] *(AU/mL)*	*Total activity (AU)*	*Activity recovered (%)*
Culture supernatant	1,000	200	2.0×10^5	100
n-BuOH extraction (leave in H_2O)	10	12,800	1.3×10^5	65
$CHCl_3$ extraction (leave in 1:1/MeOH:$CHCl_3$)	1	800	8.0×10^2	0.4
Sephadex LH-20 (leave in MeOH)	1.5	400	6.0×10^2	0.3

[a] Activity tested against LV17C(pMG36e).

2.5.3. Growth of BrcA in *C. piscicola* Using SASM Defined Medium[338,339]

Very recently, the work of Schaefer and co-workers on the characterization of the binding site of an analog of the widely studied antibiotic Lilly LY333328 in stable isotope-labeled *S. aureus* was reported in the literature.[339] In that study, labeling of the mature peptidoglycan of cell walls and whole cells of *S. aureus* was achieved using a SASM defined medium containing L-[ε-^{15}N]lysine and [1-^{13}C]glycine or D-[1-^{13}C]alanine. Preliminary studies for the production of BrcA using an unlabeled SASM defined medium were performed. Mass spectral analysis and activity assay against LV17C(pMG36e) demonstrated that the mature BrcA is produced when a culture of pJKM56 is grown in this defined medium. The antimicrobial activity results obtained at each step of the purification process are reported in Table 13. The total activity recovered when the SASM medium is used is not as good as the activity obtained with the modified CAA medium. The loss of peptide is also greater using this defined medium. Even though better than the Celtone®-U complete medium, the SASM defined medium does not compete with the original modified CAA medium. The unsatisfying results obtained led us to pursue a different approach for the production of labeled BrcA and BrcB.

Table 13. Purification of a SASM defined medium[338,339] culture of BrcA (pJKM56)

Purification stage	*Vol (mL)*	*Activity[a] (AU/mL)*	*Total activity (AU)*	*Activity recovered (%)*
Culture supernatant	1,000	200	2.0×10^5	100
n-BuOH extraction (leave in H_2O)	10	51,200	5.1×10^5	100
$CHCl_3$ extraction (leave in 1:1/MeOH:$CHCl_3$)	1	51,200	5.1×10^4	10
Sephadex LH-20 (leave in MeOH)	1.5	400	6.0×10^2	0.1

[a] Activity tested against LV17C(pMG36e).

2.6. Expression of BrcA and BrcB in *E. coli* for Facile Isotopic Labeling

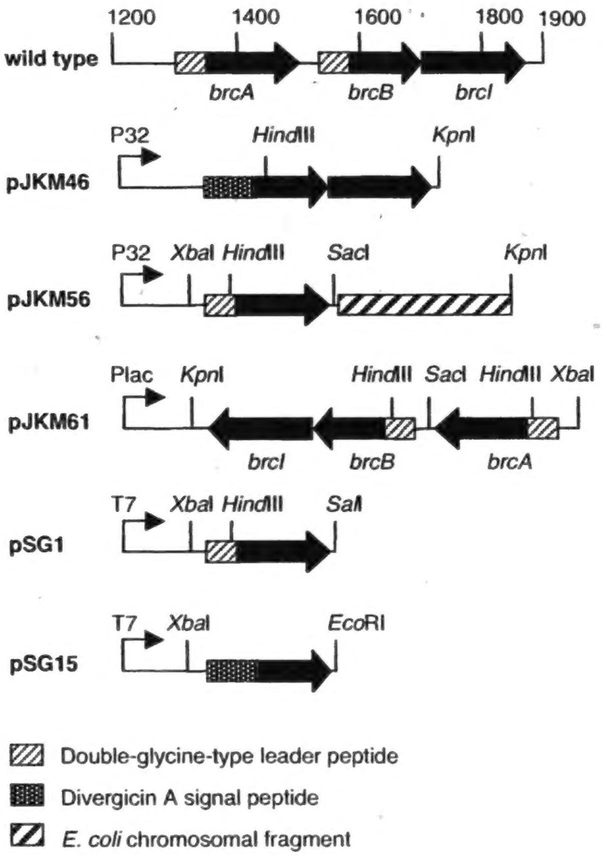
Although universal labeling of bacteriocins with ^{13}C and ^{15}N for NMR studies can be achieved in Gram-positive organisms using modified SAMS medium[339] or complex labeled media laboriously derived from blue-green algae (*Anabaena* sp.),[336] expression in *E. coli* permits facile isotopic labeling using defined media and simple commercial precursors. In an effort to produce large quantities of labeled BrcA and BrcB for NMR studies, separate expression clones of both peptides were first constructed in *E. coli*. The oligonucleotides used for primer extension in this study are listed in Table 14.

Table 14. Oligonucleotides utilized in this study

Primer	***Sequence***	***Description***
BrcA rev	5'-TACTGTACCAGCACCAATTC-3'	Complementary to reverse BrcA nucleotide sequence[277]
APO9	5'-TACTTCCGATAGCTCCTG-3'	
Primer 71	5'-ATATTCTAGATTGGAGGTTGGTATATATG-3'	Complementary to *Xba*I site of pT712
Primer 72	5'-ATATGAATTCGTATAGTTTTTACCATTGAT-3'	Complementary to *Eco*RI site of pT712
SG61331	5'-TTCCCCTCTAGAAATAATTTT-3'	Complementary to *Xba*I site of pTYB11, primer for intein forward
SG61334	5'-ATATGTCGACCTAGTTACCTAATAATCCACC-3'	Complementary to *Sal*I site of pMAL™-p2X and reverse BrcA nucleotide sequence
SG61341	5'-CAACATTTCCCCAATTTATTTTGTTCTGTACAACAACCTGGG-3'	Complementary to nucleotides 6502-6483 of pTYB11 and forward BrcB nucleotide sequence.[277] Reverse of primer SG61342
SG61342	5'-CCCAGGTTGTTGTACAGAACAAAATAAATTGGGGAAATGTTG-3'	Complementary to nucleotides 6502-6483 of pTYB11 and forward BrcB nucleotide sequence
SG61343	5'-ATATGTCGACTTACCATTGATCCCAAATACT-3'	Complementary to *Sal*I site of pTYB11 and reverse BrcB nucleotide sequence
SG61351	5'-TACAGTTCAAAAGATTGTCTA-3'	Complementary to BrcA nucleotide sequence

The construction of plasmid pSG1 for expression in *E. coli* was achieved by cloning the *dvn::brcA* gene in conjunction with the gene encoding BrcA into the *Xba*I and *Sal*I sites of pT712. Plasmid pT712 was chosen for its strong T7 promoter that promotes overexpression of the desired peptides (Figure 24). Attempts to ligate *dvn::brcB* gene and the gene encoding BrcB into pT712 digested with only *Sac*I were unsuccessful as the genes were inserted in all cases in the wrong direction (data not shown). To construct plasmid pSG15, the *dvn::brcB* gene and the gene encoding BrcB were cloned into the *Xba*I and *Eco*RI sites of pT712. DNA sequencing confirmed that the correct constructs were made.

Figure 24. Genetic organization of different expression clones of BrcA and BrcB in *C. piscicola* (pJKM46, pJKM56 and pJKM61) and in *E. coli* (pSG1 and pSG15)


1200
1400
1600
1800
1900
wild type
brcA
brcB
brcI
P32
HindIII
KpnI
pJKM46
P32
XbaI
HindIII
SacI
KpnI
pJKM56
Plac
KpnI
HindIII
SacI
HindIII
XbaI
pJKM61
brcI
brcB
brcA
T7
XbaI
HindIII
SalI
pSG1
T7
XbaI
EcoRI
pSG15
▨ Double-glycine-type leader peptide
▦ Divergicin A signal peptide
▨ E. coli chromosomal fragment

Although the expression clones pSG1 and pSG15 in *E. coli* were shown to contain the correct sequence, initial extraction of peptide fractions gave sample mixtures that showed slight activity by themselves without requiring the complementary peptide BrcA or BrcB component. MALDI-TOF mass spectrometry of both the *n*-butanol and chloroform extracts, as well as of fractions of these that were separated by HPLC, showed no signals for the parent BrcA or BrcB. Typical mass results obtained for pSG1 and pSG15 from HPLC purification for the fractions having weak activity against the indicator strain (no requirement for complementary peptide) are reported in Table 15. Depending on fermentation and extraction procedures, peptide fragments varying in size and distribution were obtained. These data indicate that in each case, the desired BrcA or BrcB are degraded into small fragments when produced in *E. coli*.

Table 15. MALDI-TOF MS from pSG1 and pSG15[a]

Plasmid	*Retention time on HPLC (min)*	*MW (Da)*	*Possible cut peptide (aa)*
pSG1	18.2-19.0	1288.95	---
		1302.87	---
		2758.56	---
		3038.77	3036.58 (9-44)
			3037.66 (23-58)
			3039.63 (18-53)
			3039.67 (6-41)
pSG15	13.4-16.0	1568.91	---
		1669.97	---
		1754.01	---
		2124.23	---
		2223.37	---
		2397.52	2398.85 (1-28)
			2398.88 (12-40)
		2407.47	---
		2533.55	2531.99 (4-34)

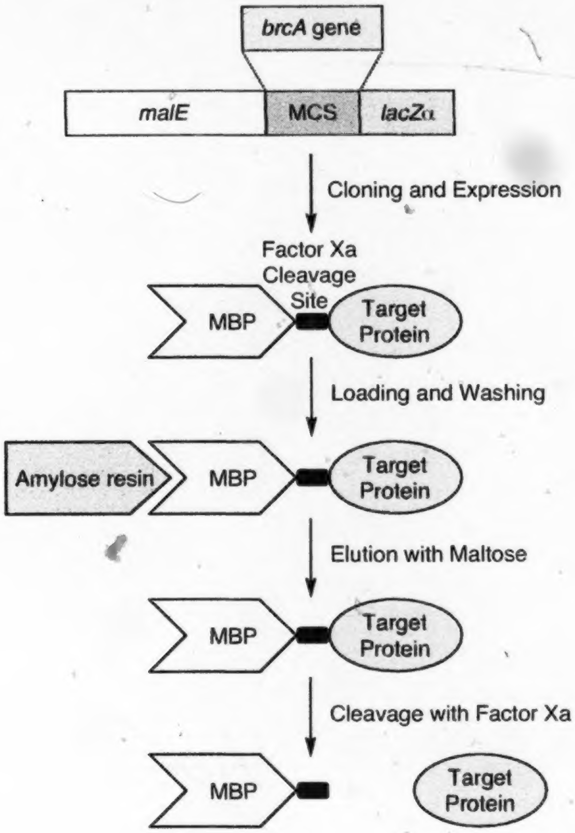
[a] Data obtained after HPLC of the *n*-BuOH extract of the French press cells of pSG1 and pSG15.

Clearly, use of the *sec* leader is insufficient to obtain relatively large bacteriocins (*i.e.* 59 amino acids) intact in *E. coli*, and approaches using large fusion proteins (*e.g.* maltose-binding protein (MBP) fusion[267] and intein protein fusion) are likely to be successful.

2.7. Expression of BrcA as a MBP Fusion in *E. coli* (pSG618422)

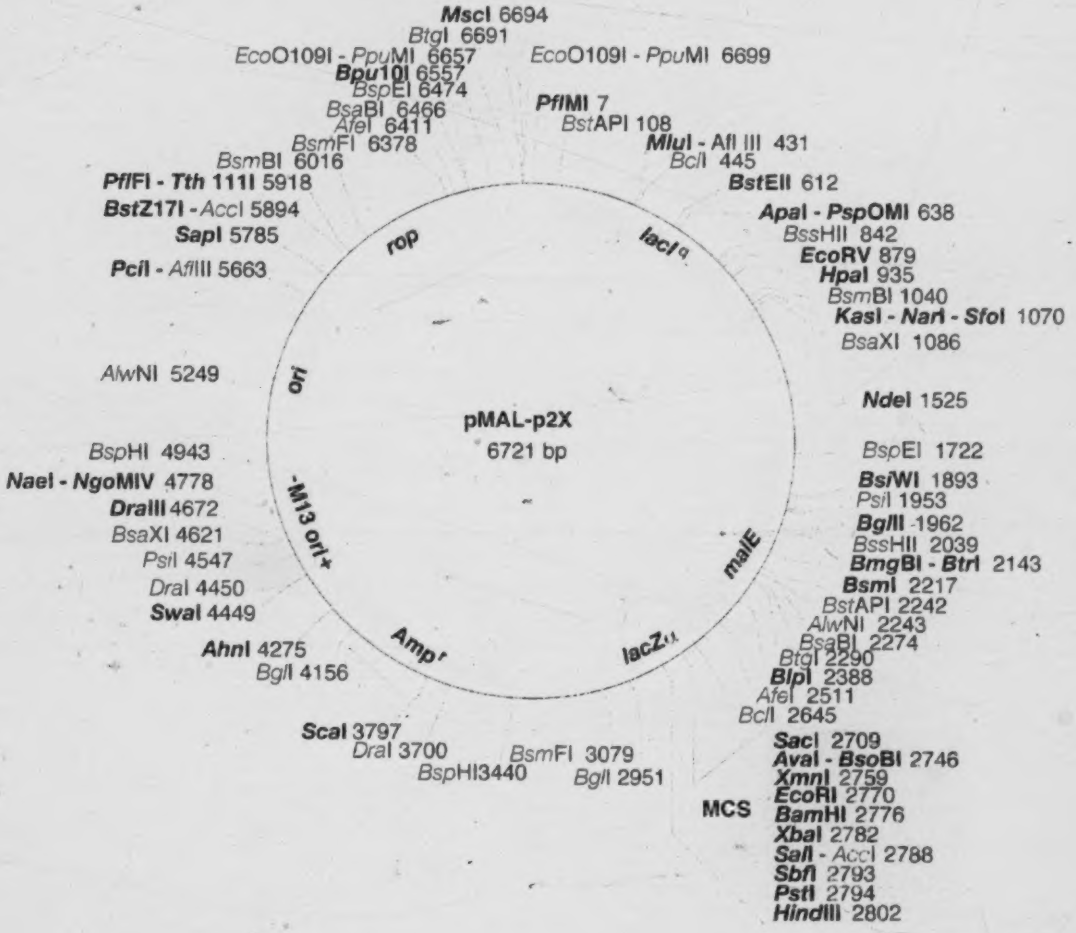
To construct a MBP fusion of BrcA, the pMAL™ system was used. In this system the cloned gene is inserted into the multiple cloning site (MCS) of a pMAL vector downstream from the *malE* gene, which encodes the MBP (Figure 25). The MCS, also called polylinker, contains a cluster of unique restriction endonuclease sites that allows for insertion of the desired gene into the vector. By having many different restriction enzyme recognition sites very near to each other, MCSs offer many choices in regard to which enzymes to use to prepare the plasmid and insert for cloning. A strong P_{tac} promoter is used in conjunction with the *malE* translation initiation signals to express large quantities of the cloned sequences. The reliable expression (100 mg/L in more than 75% of the cases tested), the enhanced solubility of the fusion proteins expressed in *E. coli*, and the one-step purification technique (maltose column) represent some of the advantages of the pMAL system. Another advantage of the pMAL system is the presence of a *lacZ*α (truncated *lacZ*) gene downstream from the MCS, which provides a simple α-complementation method to determine if DNA has been inserted into the vector. The fragment *lacZ*α can be complemented by the remaining portion of the *lacZ* encoded by the host gene *lacZ*β to produce an active enzyme capable of converting the substrate Xgal to a blue product. When disrupted by insertion of the gene of interest, the *lacZ*α is not complemented and does not produce the active enzyme. The colonies remain white on plates containing Xgal.

Figure 25. Schematic representation of the pMAL system


brcA gene
malE
MCS
lacZα
Cloning and Expression
Factor Xa Cleavage Site
MBP
Target Protein
Loading and Washing
Amylose resin
MBP
Target Protein
Elution with Maltose
MBP
Target Protein
Cleavage with Factor Xa
MBP
Target Protein

Cloning experiments were done using the pMAL™-p2X vector (Figure 26). As it contains the normal *malE* signal sequence, this plasmid expresses the desired construct in the periplasm. Vector pMAL™-p2X was chosen for several reasons. Designed to allow blue-to-white screening for inserts on Xgal by inactivation of the β-galactosidase α-fragment activity of the *malE-lacZα* fusion, this plasmid facilitates the identification of the clone or interest. It is also characterized by its four amino acid recognition sequence (Ile-(Glu or Asp)-Gly-Arg) encoding the cleavage site for Factor Xa, which allows isolation of the desired peptide from the MBP. pMAL^{TM}-p2X is the vector of choice when the peptide of interest is a secreted peptide.

Figure 26. Restriction map of pMAL^{TM}-p2X

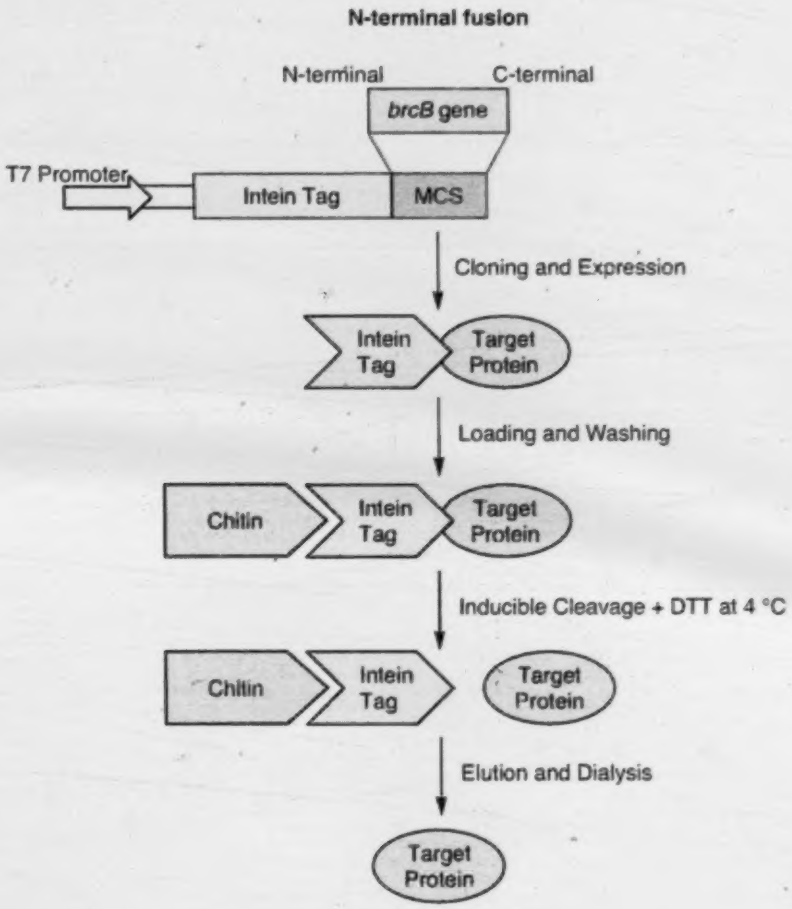

MscI 6694
BtgI 6691
EcoO109I - PpuMI 6657
EcoO109I - PpuMI 6699
Bpu10I 6557
BspEI 6474
PflMI 7
BsaBI 6466
AfeI 6411
BstAPI 108
BsmFI 6378
MluI - Afl III 431
BsmBI 6016
BclI 445
PflFI - Tth 111I 5918
BstEII 612
BstZ17I - AccI 5894
ApaI - PspOMI 638
SapI 5785
BssHII 842
EcoRV 879
PciI - AflIII 5663
HpaI 935
BsmBI 1040
KasI - NarI - SfoI 1070
BsaXI 1086
AlwNI 5249
NdeI 1525
pMAL-p2X
6721 bp
BspHI 4943
BspEI 1722
NaeI - NgoMIV 4778
BsiWI 1893
DraIII 4672
PsiI 1953
BsaXI 4621
BglII 1962
PsiI 4547
BssHII 2039
BmgBI - BtrI 2143
DraI 4450
BsmI 2217
SwaI 4449
BstAPI 2242
AlwNI 2243
AhnI 4275
BsaBI 2274
BglI 4156
BtgI 2290
BlpI 2388
AfeI 2511
BclI 2645
ScaI 3797
SacI 2709
DraI 3700
BsmFI 3079
AvaI - BsoBI 2746
BspHI3440
BglI 2951
XmnI 2759
EcoRI 2770
MCS
BamHI 2776
XbaI 2782
SalI - AccI 2788
SbfI 2793
PstI 2794
HindIII 2802
rop
lacIq
ori
M13 ori+
malE
Ampr
lacZα

The construction of plasmid pSG618422 for expression of a MBP fusion of BrcA in *E. coli* was achieved by cloning the gene encoding BrcA (isolated from pJKM56) into the *Sal*I and *Xmn*I sites of pMAL^{TM}-p2X (Figure 26). The blunt end technique using the *Xmn*I endonuclease was chosen to avoid the addition of any vector-derived residues to the desired BrcA peptide. To create the blunt-ended fragment to insert into the *Xmn*I site of the pMAL^{TM}-p2X vector, the PCR technique using primers that give a 5' blunt end and a convenient 3' sticky end was chosen. The gene encoding BrcA was isolated from pJKM56 and amplified using primers SG61351 and SG1334, which created the required 5' blunt end and the 3' sticky end respectively. DNA sequencing confirmed that the correct construct was made.

2.8. Expression of BrcB as an Intein Protein Fusion in *E. coli* (pSG619685)

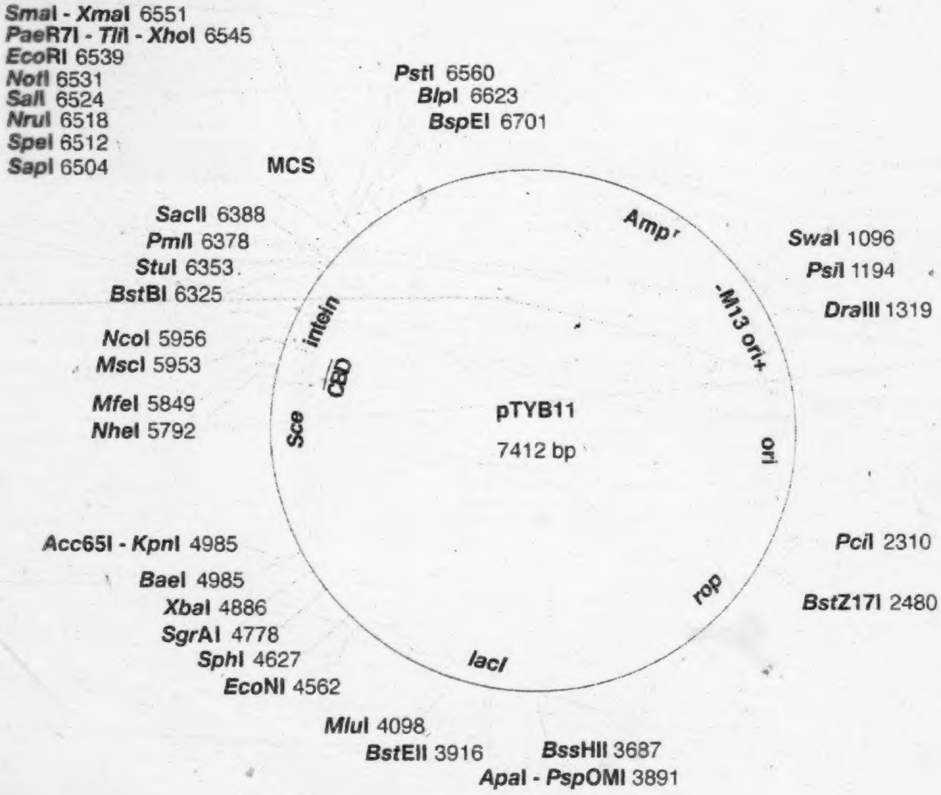
The specific protease Factor Xa required to cleave the peptide of interest from MBP is very expensive. In order to decrease the cost of production of BrcB, a novel protein purification system, IMPACT-CN (Intein Mediated Purification with an Affinity Chitin-binding Tag), was used to construct an intein protein fusion of BrcB (Figure 27). The IMPACT-CN system uses the self-cleavage activity of a protein splicing element, the intein, to isolate the target peptide from the affinity tag. The method presents several advantages including rapid and simple purification of the native peptide without extra vector-derived residues, high affinity of the chitin-binding domain (CBD) to reduce non-specific binding, and easy separation of the desired peptide from the affinity tag by using inexpensive thiol reagents (*e.g.* 1,4-dithiothreitol, β-mercaptoethanol or cysteine).

Figure 27. Schematic representation of the IMPACT-CN system with N-terminus cleavable intein tag


N-terminal fusion
N-terminal
C-terminal
brcB gene
T7 Promoter
Intein Tag
MCS
Cloning and Expression
Intein Tag
Target Protein
Loading and Washing
Chitin
Intein Tag
Target Protein
Inducible Cleavage + DTT at 4 °C
Chitin
Intein Tag
Target Protein
Elution and Dialysis
Target Protein

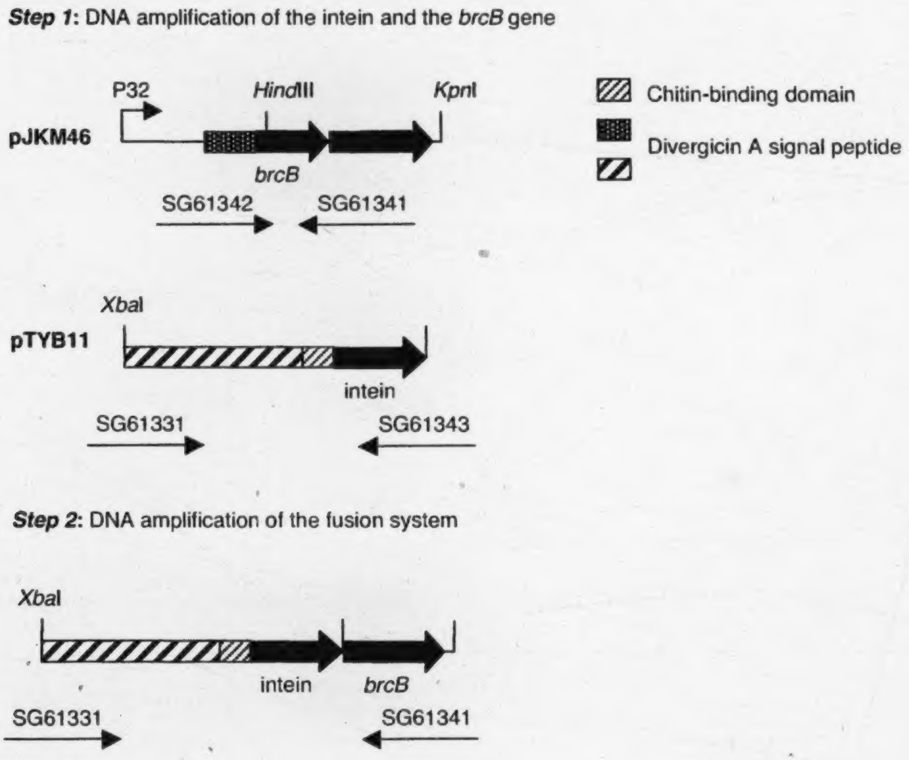
The cloning vector that was chosen for the construction of the intein protein fusion of BrcB (pSG619685) is pTYB11, a N-terminal fusion plasmid (Figure 28). Plasmid pTYB11 is characterized by its strong T7/*lac* promoter, which provides stringent control of the fusion gene expression. All other cloning vectors are known to yield target proteins with extra residues added to their N-terminus.

Figure 28. Restriction map of pTYB11


SmaI - XmaI 6551
PaeR7I - TliI - XhoI 6545
EcoRI 6539
NotI 6531
SalI 6524
NruI 6518
SpeI 6512
SapI 6504
MCS
PstI 6560
BlpI 6623
BspEI 6701
SacII 6388
PmlI 6378
StuI 6353
BstBI 6325
Amp^r
SwaI 1096
PsiI 1194
DraIII 1319
M13 ori+
NcoI 5956
MscI 5953
MfeI 5849
NheI 5792
intein
CBD
Sce
pTYB11
7412 bp
ori
Acc65I - KpnI 4985
BaeI 4985
XbaI 4886
SgrAI 4778
SphI 4627
EcoNI 4562
PciI 2310
rop
BstZ17I 2480
lacI
MluI 4098
BstEII 3916
BssHII 3687
ApaI - PspOMI 3891

To construct plasmid pSG619685 for expression of an intein protein fusion of BrcB using the IMPACT-CN system a novel cloning strategy involving double PCR experiments was developed (Figure 29). PCR experiments using combinations of primers SG61342 and SG61343, and SG61331 and SG61341 (Table 14) were first performed to amplify the *brcB* gene fusion from pJKM46 and the intein fragment of pTYB11 individually. The resulting PCR fragments could be fused as the 3' end of the intein gene and the 5' end of the *brcB* gene were amplified using primers SG61341 and SG61342 that are complementary to each other. The individual PCR fragments were then combined and amplified as a fusion system using primers SG61331 and SG61343. Cloning of the amplified fusion system into the *Xba*I and *Sal*I sites of pTYB11 was then conducted to afford pSG619685. DNA sequencing confirmed that the fusion system was inserted into pTYB11. However, complete DNA sequence could not be obtained to absolutely confirm that no mutations occurred during the cloning experiments. Studies are currently underway to verify if BrcB is expressed in such a system.

Figure 29. Cloning strategy for the construction of pSG619685


Step 1: DNA amplification of the intein and the brcB gene
P32
HindIII
KpnI
pJKM46
brcB
SG61342
SG61341
Chitin-binding domain
Divergicin A signal peptide
XbaI
pTYB11
intein
SG61331
SG61343
Step 2: DNA amplification of the fusion system
XbaI
intein
brcB
SG61331
SG61341

3. Conclusions

Bacteriocins of lactic acid bacteria were studied as potential inhibitors of the biosynthesis of peptidoglycan. Piscicolin 126, carnobacteriocins B1 and BM1, and a new bacteriocin were isolated from *Carnobacterium piscicola* 307 and 682, characterized by mass spectrometry analysis and amino acid sequencing. With the ultimate goal of determining the structure and understanding the mechanism of action of the two-peptide bacteriocin BrcC, an improved purification method was developed for BrcC using *n*-butanol and chloroform extraction. Mass spectral characterization of the two components, brochocin A (BrcA) and brochocin B (BrcB), showed that both are excreted into the medium by *B. campestris* as mature peptides of 59 and 43 amino acids, respectively. Purification from expression clones in *C. piscicola* LV17C by the new protocol showed that BrcA is expressed as the mature 59 amino acid peptide, but that BrcB is produced by *C. piscicola* as a fragment, BrcB(10-43), which has been cleaved at an internal Gly-Gly site. This fragment is not antimicrobial by itself, but in combination with BrcA displays the full activity of the BrcC complex. Circular dichroism measurements revealed a high β-sheet content in the secondary structure of both BrcA and BrcB(10-43), as well as in a 1:1/BrcA:BrcB(10-43) mixture. Separate expression clones of BrcA and BrcB were also constructed in *E. coli*, but these only produced multiple fragments of the desired peptides with little or no activity. Various culture media (peptone from cyanobacterium *Anabaena* sp. ATCC 27899, Celtone®-U and SASM defined medium) were also experimented in hope of labeling BrcA and BrcB, but peptides with poor activity were recovered after growth. A maltose-binding protein fusion of BrcA (pSG618422) and an intein protein fusion of BrcB (pSG619685) were constructed in *E. coli* to produce large quantities of labeled peptides. A complete DNA sequence confirmed that the gene encoding BrcA was correctly fused to the MBP. These fusion systems set the stage for expression in *E. coli* and facile labeling for NMR studies.

CHAPTER 3. Experimental Procedures

GENERAL METHODS FOR CHEMICAL SYNTHESES

1. Reagents, solvents and solutions

All reactions involving air or moisture sensitive reactants were done under a positive pressure of dry argon using oven-dried glassware. Reagents and solvents were reagent grade and used as supplied unless otherwise stated. For anhydrous reactions, solvents were dried according to Perrin *et al.* and Vogel.[340,341] Tetrahydrofuran and diethyl ether were distilled over sodium and benzophenone under an atmosphere of dry argon. Acetonitrile, dichloromethane, methanol, pyridine and triethylamine were distilled over calcium hydride. Removal of solvent was performed under reduced pressure below 40 °C using a Büchi rotary evaporator, followed by evacuation (< 0.1 mm Hg) to constant sample weight. Deionized water was obtained from a Milli-Q reagent water system (Millipore Co., Milford, MA). Unless otherwise specified, solutions of NH_4Cl, $NaHCO_3$, HCl, NaOH, and KOH refer to aqueous solutions. Brine refers to a saturated aqueous solution of NaCl.

All reagents employed were of American Chemical Society (ACS) grade or finer and were used without further purification unless otherwise stated. Copper (I) bromide-dimethyl sulfide complex ($CuBr \bullet Me_2S$) was either used fresh from commercial sources or recrystallized from old bottles.[342] Dimethyl acetylenedicarboxylate (DMAD) was distilled at reduced pressure before use (95-98 °C/19 mm Hg). Hexamethylphosphoramide (HMPA) was dried by stirring with calcium hydride under argon for 36 h, followed by distillation at reduced pressure and was stored over molecular sieves.

2. Purification techniques

All reactions and fractions from column chromatography were monitored by thin layer chromatography (TLC) using glass plates with a UV fluorescent indicator (normal SiO_2, Merck 60 F_{254}; reversed-phase, Merck RP-8 and RP-18 F_{254S}). One or more of the following methods were used for visualization: UV absorption by fluorescence quenching; iodine staining; (phosphomolybdic acid:ceric sulfate:sulfuric acid:H_2O/10 g:1.25 g:12 mL:238 mL) spray; and 50% sulfuric acid spray. Flash chromatography was performed according to the method of Still *et al.*[343] using Merck type 60, 230-400 mesh silica gel.

High pressure liquid chromatography (HPLC) was performed on either a Beckman System Gold instrument equipped with a model 166 variable wavelength UV detector and an Altex 210A injector with a 100 or 500 μL sample loop, or on a Rainin instrument equipped with a Rainin UV-1 detector set at 220 nm and an injector fitted with a 1 mL sample loop. The columns were Waters Nova-Pak cartridges (reversed-phase 8NVC18 4 μm C_{18} column) and Waters Resolve cartridges (reversed-phase PrePak C_{18} column, 125 Å, 10 μm, 25 x 100 mm). All HPLC solvents were prepared fresh daily and filtered with a Millipore filtration system under vacuum before use.

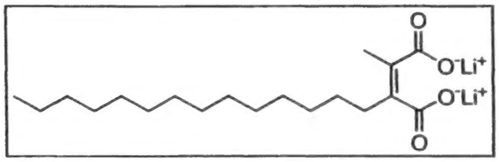
3. Instrumentation for compound characterization

Melting points are uncorrected and were determined on a Thomas-Hoover or a Büchi oil immersion apparatus using open capillary tubes. Optical rotations were measured on a Perkin Elmer 241 polarimeter with a microcell (10 cm, 1 mL) at ambient temperature and are reported in units of 10^{-1} deg cm^2 g^{-1}. All optical rotations reported were referenced against air and were measured at the sodium D line and values quoted are valid within ±1°. Infrared spectra (IR) were recorded on a Nicolet Magna 750 or a 20SX FT-IR spectrometers. Cast refers to the evaporation of a solution on a NaCl plate. Mass spectra (MS) were recorded on a Kratos AEIMS-50 (high resolution (HRMS), electron impact ionization (EI), MS-9 (fast atom bombardment with argon (FAB), and Micromass ZabSpec Hybrid Sector-TOF positive mode electrospray ionization ((ES), 0.5% solution of formic acid in MeCN:H_2O/1:1) instruments. Cleland matrix used in FAB spectra refers to a 5:1 mixture of dithiothreitol and dithioerythritol. Microanalyses were obtained using a Perkin Elmer 240 or Carlo Erba 1108 elemental analyzers.

Nuclear magnetic resonance (NMR) spectra were obtained on Bruker AM-300 and WM-360 or Inova Varian 300, 500 and 600 MHz spectrometers. ^{1}H NMR chemical shifts are reported in parts per million (ppm) downfield relative to tetramethylsilane (TMS) using the residual proton resonance of solvents as reference: $CDCl_3$ δ 7.24, CD_2Cl_2 δ 5.32, D_2O δ 4.72, and CD_3OD δ 3.30. ^{13}C NMR chemical shifts are reported relative to $CDCl_3$ δ 77.0, CD_2Cl_2 δ 53.8, and CD_3OD δ 49.0. Selective homonuclear decoupling, shift correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), and attached proton test (APT) were used for signal assignments of all carbohydrates. ^{1}H NMR data are reported in the following order: multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; qn, quintet and m, multiplet), number of protons, coupling constant (*J*) in Hertz (Hz) and assignment. When appropriate, the multiplicity is preceded by br, indicating that the signal was broad. All literature compounds had IR, ^{1}H NMR, and mass spectra consistent with the assigned structures. More detailed analysis of ^{1}H NMR and ^{13}C NMR spectra was done for all known compounds.

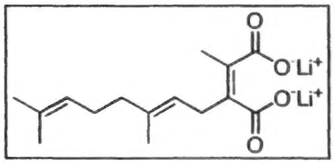
EXPERIMENTAL DATA FOR COMPOUNDS

***Chaetomellic acid A dilithium salt* (13).**



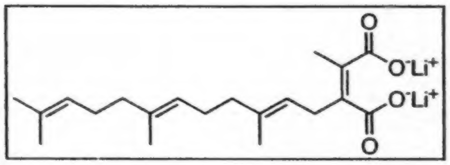
A solution of 1 N LiOH (0.3 mL, 2.1 eq) was added to diester **19** (50 mg, 0.14 mmol) in a THF:H_2O/1:1 solution (2 mL). The reaction mixture was stirred at rt for 3 days. The solvent was removed under reduced pressure and the remaining solid was dissolved in H_2O. Non-polar impurities including unreacted starting material were removed by extraction with Et_2O. Freeze-drying of the aqueous layer gave **13** (47 mg, 99%) as a white solid: IR (KBr) 3440, 2921, 2851, 1555, 1438 cm^{-1}; ^{1}H NMR (CD_3OD, 400 MHz) δ 2.24 (t, 2H, *J* = 7.8 Hz, $CH_2C\underline{H}_2C{=}C$), 1.83 (s, 3H, $C\underline{H}_3C{=}C$), 1.47 (m, 2H, $C\underline{H}_2CH_2C{=}C$), 1.28 (br m, 22H, $(C\underline{H}_2)_{11}$), 0.89 (t, 3H, *J* = 6.8 Hz, $C\underline{H}_3$-chain); ^{13}C NMR (CD_3OD, 75 MHz) δ 180.2 and 179.9 (2x$\underline{C}{=}O$), 139.6 and 132.8 ($\underline{C}{=}\underline{C}$), 33.1, 31.6, 31.1, 30.5, 30.4, 30.3, 29.5 and 23.7 ($(\underline{C}H_2)_{13}$), 16.3 and 16.2 (2x$\underline{C}H_3$); MS (FAB Cleland) *m/z* (relative intensity) 339 [MH^+, 9%].

***(Z)-2-Geranyl-3-methylbutenedioic acid dilithium salt* (14).**



A solution of 1.75 N LiOH (0.58 mL, 5.3 eq) was added to diester **20** (57 mg, 0.19 mmol) in a THF:H_2O/1:1 solution (2.3 mL). The reaction mixture was stirred at rt for 2 days. The solvent was removed under reduced pressure and the remaining solid was dissolved in H_2O. Non-polar impurities including unreacted starting material were removed by extraction with Et_2O. Freeze-drying of the aqueous layer gave **14** (48 mg, 90%) as a white solid: IR ($CHCl_3$ cast) 2920, 1590, 1543, 1435, 1401 cm^{-1}; ^1H NMR (CD_3OD, 360 MHz) δ 5.24-5.21 and 5.10-5.07 (2m, 2H, 2xC=C$\underline{H}$), 2.99 (d, 2H, *J* = 6.6 Hz, C=CHC$\underline{H}_2$C=C), 2.10-1.90 (m, 4H, (C$\underline{H}_2$)$_2$), 1.83 (s, 3H, C$\underline{H}_3$C=CCO), 1.67 (s, 3H, C$\underline{H}_3$-chain), 1.61 (s, 6H, 2xC$\underline{H}_3$-chain); ^{13}C NMR (CD_3OD, 75 MHz) δ 180.5 and 179.5 (2x$\underline{C}$=O), 137.5, 135.9, 133.8 and 132.3 (4x$\underline{C}$=C), 125.5 and 124.1 (2xC=$\underline{C}$H), 33.1, 30.1 and 27.6 (3xC$\underline{H}_2$), 25.9 ($\underline{C}H_3$C=CCO), 23.6 and 16.5 (3x$\underline{C}H_3$-chain); MS (FAB Cleland) *m/z* (relative intensity) 279 [MH^+, 9%].

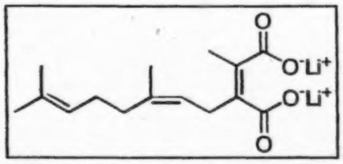
***(Z)-2-Farnesyl-3-methylbutenedioic acid dilithium salt* (15).**



A solution of 1.75 N LiOH (0.46 mL, 5.3 eq) was added to diester **21** (55 mg, 0.15 mmol) in a THF:H_2O/1:1 solution (2.2 mL). The reaction mixture was stirred at rt for 3 days. The solvent was removed under reduced pressure and the remaining solid was dissolved in H_2O. Non-polar impurities including unreacted starting material were removed by extraction with Et_2O. Freeze-drying of the aqueous layer gave **15** (54 mg, 99%) as a white solid: IR ($CHCl_3$ cast) 2920, 1590, 1543, 1435, 1401 cm^{-1}; ^1H NMR (CD_3OD, 360 MHz) δ 5.25-5.20 (m, 1H, C=C$\underline{H}$), 5.10-5.06 (m, 2H, 2xC=C$\underline{H}$), 2.99 (d, 2H, *J* = 6.7 Hz, C=CHC$\underline{H}_2$C=C), 2.11-1.93 (m, 8H, 2x(C$\underline{H}_2$)$_2$), 1.83 (s, 3H, C$\underline{H}_3$C=CCO), 1.66 and 1.65 (2s, 6H, 2xC$\underline{H}_3$-chain), 1.58 (s, 6H, 2xC$\underline{H}_3$-chain); ^{13}C NMR (CD_3OD, 75 MHz) δ 180.5 and 179.5 (2x$\underline{C}$=O), 137.5, 136.2, 135.9, 134.0 and 132.4 (5x$\underline{C}$=C), 125.5, 125.2 and 123.1 (3xC=$\underline{C}$H), 40.7, 40.6, 30.1, 27.6 and 27.5

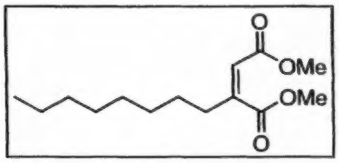
(5x$\underline{C}H_2$), 25.9 ($\underline{C}H_3C$=CCO), 17.8, 16.4, and 16.1 (4x$\underline{C}H_3$); MS (CI, NH_3) *m/z* (relative intensity) 364 [MNH_4^+, 6%].

***(Z)-2-Nerolyl-3-methylbutenedioic acid dilithium salt* (16).**



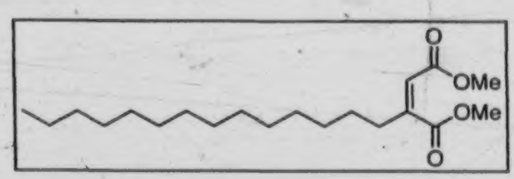
A solution of 1 N LiOH (0.53 mL, 3.5 eq) was added to diester **22** (31 mg, 0.11 mmol) in a THF:H_2O/1:1 solution (2 mL). The reaction mixture was stirred at rt for 2 days. The solvent was removed under reduced pressure and the remaining solid was dissolved in H_2O. Non-polar impurities including unreacted starting material were removed by extraction with Et_2O. Freeze-drying of the aqueous layer gave **16** (29 mg, 99%) as a white solid: IR ($CDCl_3$ cast) 2920, 1590, 1543, 1435, 1401 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 5.24-5.20 and 5.17-5.13 (2m, 2H, 2xC=C$\underline{H}$), 2.99 (d, 2H, *J* = 6.6 Hz, C=CHC$\underline{H}_2$C=C), 2.12-2.08 (m, 4H, ($C\underline{H}_2)_2$), 1.83 (s, 3H, C$\underline{H}_3$C=CCO), 1.67 and 1.61 (2s, 9H, 3xC$\underline{H}_3$-chain); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 180.5 and 179.5 (2x$\underline{C}$=O), 137.5, 135.9, 133.8 and 132.3 (4x$\underline{C}$=C), 125.5 and 124.1 (2xC=$\underline{C}$H), 33.1, 30.1 and 27.6 (3x$\underline{C}H_2$), 25.9 ($\underline{C}H_3C$=CCO), 23.6, 17.7 and 16.5 (3x$\underline{C}H_3$-chain); MS (FAB, Cleland) *m/z* (relative intensity) 279 [MH^+, 28%].

***Dimethyl (Z)-2-octylbutenedioate* (17).**



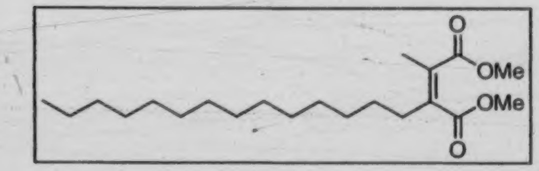
Octylmagnesium chloride (1.20 mL of a 2.0 M solution in THF, 2.4 mmol) was added dropwise to a suspension of $CuBr{\bullet}Me_2S$ (0.5 g, 2.4 mmol) in THF (12 mL) at -40 °C. The resulting yellow suspension was stirred at -40 °C for 2 h, then cooled to -78 °C, and freshly distilled DMAD (0.24 mL, 2.00 mmol) in THF (2 mL) was added dropwise to give a dark red brown mixture. After 1 h, the reaction mixture was quenched with a saturated aq. NH_4Cl solution (4 mL, adjusted to pH 8 with 10% ammonia) and allowed to warm to rt. After 30 min, the mixture was partitioned between Et_2O and H_2O. The aqueous layer was extracted with Et_2O (3x10 mL) and the combined organic extracts were washed with a saturated aq. NH_4Cl solution (40 mL) and brine (40 mL), dried (Na_2SO_4), and concentrated *in vacuo*. Further purification by flash chromatography (SiO_2, 3:2/hexane:EtOAc, R_f 0.66) gave **17** (446 mg, 87%) as a colorless oil: IR ($CDCl_3$ cast) 2927, 2856, 1730, 1652, 1436, 1371, 1265, 1197, 1167, 1100, 830 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.76 (t, 1H, J = 1.5 Hz, $C{=}C\underline{H}$), 3.78 and 3.68 (2s, 6H, $2xOC\underline{H}_3$), 2.33-2.28 (m, 2H, $CH_2C\underline{H}_2C{=}C$), 1.45 (qn, 2H, $C\underline{H}_2CH_2C{=}C$), 1.22 (br s, 10H, $(C\underline{H}_2)_5$), 0.84 (t, 3H, J = 6.8 Hz, $C\underline{H}_3$-chain); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 169.4 and 165.4 ($2x\underline{C}{=}O$), 151.0 ($\underline{C}{=}CH$), 119.0 ($C{=}\underline{C}H$), 52.2 and 51.7 ($2xO\underline{C}H_3$), 34.4, 31.8, 29.2, 29.1, 28.9, 26.9 and 22.6 ($(\underline{C}H_2)_7$), 14.0 ($\underline{C}H_3$-chain); HRMS (EI) Calcd for $C_{14}H_{24}O_4$ 256.1675, found 256.1675 $[M]^+$.

Dimethyl (Z)-2-tetradecylbutenedioate **(18).**



The reaction of tetradecylmagnesium chloride (1.20 mL of a 1.0 M solution in THF, 1.2 mmol), CuBr•Me$_2$S (0.25 g, 1.20 mmol) in THF (6 mL), and DMAD (0.14 g, 1.00 mmol) in THF (2 mL) was performed as described for the synthesis of **17**. Further purification by flash chromatography (SiO$_2$, 9:1/petroleum ether: Et$_2$O, R$_f$ 0.26 (9:1/hexane:EtOAc)) gave **18** (289 mg, 85%) as a white solid: mp 51-52 °C; IR (CDCl$_3$ cast) 2948, 2917, 2850, 1730, 1718, 1653, 1375, 1261, 1199, 1170 cm^{-1}; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 5.78 (t, 1H, J = 1.5 Hz, C=C$\underline{H}$), 3.81 and 3.70 (2s, 6H, 2xOC$\underline{H}_3$), 2.32 (td, 2H, J = 7.7 and 1.5 Hz, CH$_2$C$\underline{H}_2$C=C), 1.47 (qn, 2H, C$\underline{H}_2$CH$_2$C=C), 1.24 (br s, 22H, (C$\underline{H}_2$)$_{11}$), 0.86 (t, 3H, J = 6.6 Hz, C$\underline{H}_3$-chain); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 169.5 and 165.5 (2x$\underline{C}$=O), 151.1 ($\underline{C}$=CH), 119.0 (C=$\underline{C}$H), 52.3 and 51.8 (2xO$\underline{C}$H$_3$), 34.5, 32.0, 29.73, 29.71, 29.69, 29.64, 29.5, 29.4, 29.3, 29.0 and 22.7 ($\underline{C}$H$_2$)$_{13}$), 14.2 ($\underline{C}$H$_3$-chain); HRMS (EI) Calcd for C$_{20}$H$_{36}$O$_4$ 340.2614, found 340.2614 [M]$^+$; Anal. Calcd for C$_{20}$H$_{36}$O$_4$: C, 70.55; H, 10.66. Found: C, 70.63; H, 10.71.

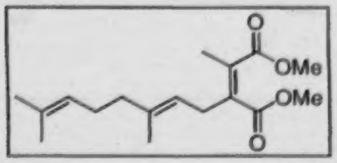
Chaetomellic acid A dimethyl ester **(19).**



The known diester **19**[146,344,345] was prepared using the method described by Ratemi.[146] Tetradecylmagnesium chloride (3.6 mL of a 1.0 M solution in THF, 3.6 mmol) was added dropwise to a suspension of CuBr•Me$_2$S

(0.75 g, 3.6 mmol) in THF (18 mL) at -40 °C. The resulting yellow suspension was stirred at -40 °C for 2 h, then cooled to -78 °C, and freshly distilled DMAD (0.42 g, 0.36 mL, 3.0 mmol) in THF (6 mL) was added dropwise to give a dark red brown mixture. After 40 min, a HMPA:THF/1:1 solution (6 mL) was added dropwise, and the reaction mixture was stirred for 45 min. Methyl iodide (1.08 g, 0.47 mL, 7.5 mmol) in THF (6 mL) was then added dropwise and the reaction mixture was stirred for 5 min at -78 °C. The reaction mixture was warmed to rt. After 18.5 h, the reaction mixture was quenched with a saturated aq. NH_4Cl solution (6 mL, adjusted to pH 8 with 10% ammonia). After 30 min, the mixture was filtered through a pad of Celite®. The aqueous layer was extracted with EtOAc, and the combined organic extracts were washed with a saturated aq. NH_4Cl solution, H_2O, and brine, dried (Na_2SO_4), and concentrated *in vacuo*. Further purification by flash chromatography (SiO_2, 4:1/petroleum ether:Et_2O) gave **19** (808 mg, 76%) as a colorless oil: IR ($CHCl_3$ cast) (lit.[146,344]) 2924, 2853, 1725, 1644, 1434 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) (lit.[146,344-346]) δ 3.74 and 3.73 (2s, 6H, $2xOC\underline{H}_3$), 2.31 (t, 2H, J = 7.5 Hz, $CH_2C\underline{H}_2C{=}C$), 1.93 (s, 3H, $C\underline{H}_3C{=}C$), 1.42 (qn, 2H, $C\underline{H}_2CH_2C{=}C$), 1.25-1.24 (br m, 22H, $(C\underline{H}_2)_{11}$), 0.86 (t, 3H, J = 6.5 Hz, $C\underline{H}_3$-chain); ^{13}C NMR ($CDCl_3$, 75 MHz) (lit.[146]) δ 169.9 and 169.1 ($2x\underline{C}{=}O$), 139.7 and 131.5 ($\underline{C}{=}\underline{C}$), 52.1 and 52.0 ($2xO\underline{C}H_3$), 31.9, 30.1, 29.6, 29.5, 29.4, 29.3, 27.7 and 22.6 ($(\underline{C}H_2)_{13}$), 14.9 and 14.0 ($2x\underline{C}H_3$); HRMS (EI) Calcd for $C_{21}H_{38}O_4$ 354.2770, found 354.2763 $[M]^+$.

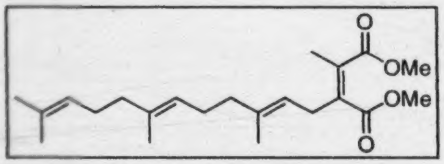
Dimethyl (Z)-2-geranyl-3-methylbutenedioate (**20**).



Methylmagnesium bromide (1.33 mL of a 3.0 M solution in THF, 4 mmol) was added dropwise to a suspension of CuBr•Me_2S (0.82 g, 4 mmol) in THF (19 mL) at -40 °C. The resulting yellow suspension was stirred at -40 °C for 2 h, then cooled to -78 °C, and freshly distilled DMAD (0.43 mL, 3.5 mmol) in THF (8 mL) was added dropwise to give a dark red brown mixture. After 40 min, a HMPA:THF/1:1 solution (8 mL) was added dropwise, which resulted in the heterogeneous mixture becoming nearly homogeneous. After 45 min, geranyl bromide (1.39 mL, 7 mmol) in THF (8 mL) was added and stirring was continued at -78 °C for 5 min. After warming to rt overnight, the reaction mixture was re-cooled to -20 °C, quenched with a saturated aq. NH_4Cl solution (8 mL, adjusted to pH 8 with 10% ammonia), and allowed to warm to rt. After 30 min, the mixture was filtered through a pad of Celite®. The aqueous layer was extracted with Et_2O, and the combined organic extracts were washed with a saturated aq. NH_4Cl solution, H_2O, and brine, dried ($MgSO_4$), and concentrated *in vacuo*. Further purification by flash chromatography (SiO_2, 4:1/petroleum ether:Et_2O, R_f 0.29) gave **20** (867 mg, 84%) as a colorless oil: IR ($CDCl_3$ cast) 2950, 1724, 1666, 1434 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 5.01-4.97 (m, 2H, 2xC=C$\underline{H}$), 3.69 and 3.68 (2s, 6H, 2xOC$\underline{H}_3$), 3.00 (d, 2H, J = 7.0 Hz, C=CHC$\underline{H}_2$C=C), 2.05-1.94 (m, 4H, (C$\underline{H}_2)_2$), 1.90 (s, 3H, C$\underline{H}_3$C=CCO), 1.61, 1.59 and 1.53 (3s, 9H, 3xC$\underline{H}_3$-chain); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 169.0 and 168.9 (2x$\underline{C}$=O), 137.9, 137.8, 131.9 and 131.3 (4x$\underline{C}$=C), 123.8 and 118.4 (2xC=$\underline{C}$H), 51.9 and 51.8

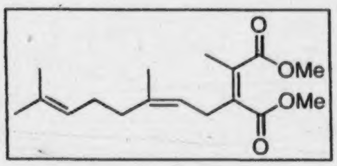
(2xOCH$_3$), 39.4 (C=CHCH$_2$C=C), 28.7 and 26.4 ((CH$_2$)$_2$), 25.5 (CH$_3$C=CCO), 15.9 and 14.9 (3xCH$_3$-chain); HRMS (EI) Calcd for $C_{17}H_{26}O_4$ 294.1831, found 294.1828 [M]$^+$.

***Dimethyl (Z)-2-farnesyl-3-methylbutenedioate* (21).**



The reaction of methylmagnesium bromide (0.67 mL of a 3.0 M solution in THF, 2 mmol), CuBr•Me$_2$S (0.41 g, 2 mmol) in THF (9.8 mL), freshly distilled DMAD (0.23 mL, 1.9 mmol) in THF (4 mL), a HMPA:THF/1:1 solution (4 mL), and farnesyl bromide (1.08 mL, 4 mmol) in THF (4 mL) was performed as described for the synthesis of **20**. Further purification by flash chromatography (SiO$_2$, 4:1/petroleum ether:Et$_2$O, R_f 0.39) gave **21** (567 mg, 82%) as a colorless oil: IR (CDCl$_3$ cast) 2949, 2920, 1725, 1643, 1434 cm^{-1}; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 5.10-5.00 (m, 3H, 3xC=CH), 3.71 and 3.70 (2s, 6H, 2xOCH$_3$), 3.03 (d, 2H, J = 6.9 Hz, C=CHCH$_2$C=C), 2.05-1.93 (m, 8H, 2x(CH$_2$)$_2$), 1.93 (s, 3H, CH$_3$C=CCO), 1.65 and 1.63 (2s, 6H, 2xCH$_3$-chain), 1.57 (s, 6H, 2xCH$_3$-chain); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 169.3 and 169.2 (2xC=O), 138.23, 138.19, 135.3, 132.1 and 131.3 (5xC=C), 124.4, 123.9 and 118.4 (3xC=CH), 52.2 and 52.1 (2xOCH$_3$), 39.7 (C=CHCH$_2$C=C), 29.0, 26.8, 26.6 and 25.7 (2x(CH$_2$)$_2$), 17.1, 16.2, 16.0, 15.3 and 15.2 (5xCH$_3$); HRMS (EI) Calcd for $C_{22}H_{34}O_4$ 362.2457, found 362.2449 [M]$^+$.

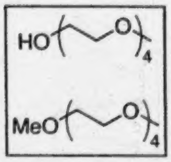
***Dimethyl (Z)-2-nerolyl-3-methylbutenedioate* (22).**



***Preparation of nerolyl bromide*:** To a solution of nerol (1.59 mL, 9 mmol) in THF (10 mL) was added dropwise a solution of phosphorous tribromide (0.36 mL, 3.77 mmol) in THF (5 mL) at -10 °C. The reaction mixture was stirred for 15 min, and concentrated *in vacuo*. The residue obtained was dissolved in a hexane:diisopropyl ether/1:1 solution (15 mL), washed with 5% aq. $NaHCO_3$, H_2O, dried (Na_2SO_4), and concentrated *in vacuo* to give crude nerolyl bromide (1.92 g, 98%) as a pale yellow oil. This product was used without further purification for the preparation of dimethyl (*Z*)-2-nerolyl-3-methylbutenedioate (**22**).

***Preparation of* 22:** The reaction of methylmagnesium bromide (1.33 mL of a 3.0 M solution in THF, 4 mmol), CuBr•Me_2S (0.82 g, 4 mmol) in THF (19 mL), freshly distilled DMAD (0.43 mL, 3.5 mmol) in THF (8 mL), a HMPA:THF/1:1 solution (8 mL), and freshly prepared nerolyl bromide (1.92 g, 8.8 mmol) was performed as described for the synthesis of **20**. Further purification by flash chromatography (SiO_2, 4:1/petroleum ether:Et_2O, R_f 0.23) gave **22** (751 mg, 73%) as a colorless oil: IR ($CDCl_3$ cast) 2951, 1725, 1644, 1434, 1265 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 5.10-4.98 (m, 2H, 2xC=C$\underline{H}$), 3.72 and 3.71 (2s, 6H, 2xOC$\underline{H}_3$), 3.03 (d, 2H, *J* = 6.9 Hz, C=CHC$\underline{H}_2$C=C), 2.05-2.02 (m, 4H, (C$\underline{H}_2$)$_2$), 1.93 (s, 3H, C$\underline{H}_3$C=CCO), 1.67, 1.66 and 1.59 (3s, 9H, 3xC$\underline{H}_3$-chain); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 169.21 and 169.20 (2x$\underline{C}$=O), 138.2, 138.1, 132.3 and 131.9 (4x$\underline{C}$=C), 124.0 and 119.2 (2xC=$\underline{C}$H), 52.2 and 52.1 (2xO$\underline{C}H_3$), 32.1 (C=CH$\underline{C}H_2$C=C), 28.7 and 26.4 ($((\underline{C}H_2)_2)$), 25.7 ($\underline{C}H_3C{=}CCO$), 23.3, 17.7 and 15.2 (3x$\underline{C}H_3$-chain); HRMS (EI) Calcd for $C_{17}H_{26}O_4$ 294.1831, found 294.1823 $[M]^+$.

***Dimethoxytetraethylene glycol* (26) *and tetraethylene glycol monomethyl ether* (28).**



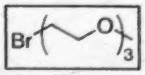
The known compound **28**[347-349] was prepared using the method described by Ercolani.[151] Sodium-dried benzene (300 mL) and sodium wire (7 g, 0.304 mol) were placed in a 500 mL three-necked round-bottomed flask. Tetraethylene glycol (**25**) (38.4 mL, 0.289 mol) was added dropwise at rt over 40 min under vigorous stirring. After complete dissolution of the sodium, methyl iodide (19.8 mL, 0.317 mol) was added dropwise over 15 min to the well stirred mixture which was then heated at 80 °C with stirring for 2 h. The reaction mixture was transferred into a separatory funnel. The upper benzenic phase was discarded, and the lower thick layer was taken up with $CHCl_3$ and filtered free from any precipitated NaI. The clear filtrate was shaken with H_2O and then separated. The aqueous solution containing the unreacted starting material was discarded. The $CHCl_3$ layer was extracted with H_2O (1 L) and the aqueous layer was repeatedly extracted with small portions of CCl_4, which selectively extracts the dimethyl derivative. The crude tetraethylene glycol monomethyl ether (16 g) was obtained as a yellow oil by distillation of the H_2O under reduced pressure. Further purification by vacuum distillation (127-130 °C/2 mm Hg) (lit.[350] bp 105-110 °C/0.03 Torr) (lit.[151] bp 110-120 °C/1.0 Torr) (lit.[351] bp 115-117 °C/0.01 Torr) (lit.[352] bp 122.85 °C/0.8 Torr) (lit.[347] bp 123-125 °C/1.0 Torr) (lit.[353] bp 125-130 °C/3.0-3.5 Torr) (lit.[354] bp 126 °C/2.0 Torr) (lit.[355] bp 158-160 °C/12x10^{1} Torr) followed by flash chromatography

(SiO_2, 5:95/MeOH:$CHCl_3$, R_f**26** 0.40 and R_f**28** 0.25) gave **26** (16.1 g, 25%) and **28** (28.9 g, 48%) as colorless oils.

Data for **26**: IR ($CHCl_3$ cast) 2875, 1109 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 3.64-3.50 (m, 16H, $(C\underline{H}_2C\underline{H}_2O)_4$), 3.35 (s, 6H, $2xOC\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 72.0, 70.7, 70.63 and 70.56 ($8x\underline{C}H_2$), 59.1 ($2xO\underline{C}H_3$).

Data for **28**: IR ($CDCl_3$ cast) 3600-3200, 2880, 1104, 753 cm^{-1} (lit.[356] IR); ^1H NMR ($CDCl_3$, 300 MHz) (lit.[357]) δ 3.72-3.51 (m, 6H, $(C\underline{H}_2C\underline{H}_2O)_4$), 3.36 (s, 3H, $OC\underline{H}_3$), 2.55 (d, 1H, J = 3.9 Hz, $O\underline{H}$); ^{13}C NMR ($CDCl_3$, 75 MHz) (lit.[357]) δ 72.5, 72.0, 70.68, 70.63 70.57 and 70.4 ($7x\underline{C}H_2$), 61.8 ($\underline{C}H_2OH$), 59.1 ($O\underline{C}H_3$); HRMS (EI) Calcd for $C_9H_{21}O_5$ 209.1389, found 209.1400 $[M+H]^+$.

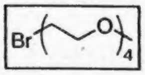
1-Bromo-3,6,9-trioxatrioctane **(29).**



The known compound **29**[152,358,359] was prepared using a bromination method described by Hayashi.[155] Triphenylphosphine (3.185 g, 12.18 mmol) in dry CH_2Cl_2 (3.5 mL) was added dropwise through an addition funnel over a period of 4 h to a well-stirred solution of commercially available triethylene glycol monomethyl ether (**27**) (2 g, 12.18 mmol) and CBr_4 (6.06 g, 18.27 mmol) in dry CH_2Cl_2 (2.5 mL) at rt. After one night, the reaction mixture was treated with pentane (95 mL), and the resulting precipitate (triphenylphosphine oxide) was removed by filtration and washed several times with pentane. The combined organic solutions were washed with 5% aq. $NaHCO_3$, brine, dried ($MgSO_4$), and concentrated *in vacuo*. Further purification by flash chromatography (SiO_2, 1:1/petroleum ether:Et_2O, R_f 0.54 (100% Et_2O)) gave **29** (2.01 g, 73%) as a colorless oil:

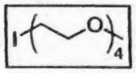
IR (neat) (lit.[152]) 2875, 1109 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) (lit.[152,358]) δ 3.77 (t, 2H, *J* = 6.3 Hz, $C\underline{H}_2CH_2Br$), 3.65-3.49 (2m, 8H, $CH_3O(C\underline{H}_2C\underline{H}_2)_2$), 3.43 (t, 2H, *J* = 6.3 Hz, $CH_2C\underline{H}_2Br$), 3.40 (s, 3H, $OC\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 71.9, 71.2, 70.62, 70.60 and 70.5 (5x$\underline{C}H_2$), 59.0 ($O\underline{C}H_3$), 30.3 ($\underline{C}H_2Br$); MS (ES+) *m/z* (relative intensity) 249.0 [MNa$^+$, 100%], 227.0 [MH$^+$, 11%].

1-Bromo-3,6,9,12-tetraoxatridecane (30).



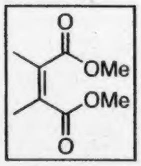
The known compound **30**[151,157] was prepared based on a bromination method described by Hayashi.[155] The reaction of triphenylphosphine (2.906 g, 11.11 mmol) in dry CH_2Cl_2 (3.2 mL), tetraethylene glycol monomethyl ether (**16**) (2.314 g, 11.11 mmol), and CBr_4 (5.528 g, 16.67 mmol) in dry CH_2Cl_2 (2.2 mL) was performed as described for the synthesis of **29**. Further purification by flash chromatography (SiO_2, 1:1/petroleum ether:Et_2O, R_f 0.38 (100% Et_2O)) gave **30** (2.22 g, 74%) as a colorless oil: IR ($CHCl_3$ cast) 2873, 1109 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.79 (t, 2H, *J* = 6.6 Hz, $C\underline{H}_2CH_2Br$), 3.66-3.51 (2m, 12H, $CH_3O(C\underline{H}_2C\underline{H}_2)_3$), 3.44 (t, 2H, *J* = 6.9 Hz, $CH_2C\underline{H}_2Br$), 3.36 (s, 3H, $OC\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 72.0, 71.3, 70.71, 70.67 and 70.60 (7x$\underline{C}H_2$), 59.1 ($O\underline{C}H_3$), 30.3 ($\underline{C}H_2Br$); MS (ES+) *m/z* (relative intensity) 295.1 [MNa$^+$, 100%].

1-Iodo-3,6,9,12-tetraoxatridecane (31).



The iodination reaction was done using the method described by Verheyden.[360] A solution of tetraethylene glycol monomethyl ether (**28**) (592 mg, 2.84 mmol), triphenylphosphine (745 mg, 2.84 mmol), and iodine (722 mg, 2.84 mmol) in anhydrous DMF (15 mL) was stirred at rt for 24 h, then evaporated to dryness. Further purification by flash chromatography (SiO_2, 100% Et_2O, R_f 0.21) gave **31** (524 mg, 58%) as a colorless oil: IR ($CHCl_3$ cast) 2871, 1107 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 3.74 (t, 2H, J = 6.9 Hz, $C\underline{H}_2CH_2I$), 3.67-3.51 (2m, 12H, $CH_3O(C\underline{H}_2C\underline{H}_2)_3$), 3.36 (s, 3H, $OC\underline{H}_3$), 3.24 (t, 2H, J = 6.9 Hz, $CH_2C\underline{H}_2I$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 72.00, 71.96, 70.68, 70.64, 70.62, 70.55 and 70.2 ($7x\underline{C}H_2$), 59.0 ($O\underline{C}H_3$), 2.9 ($\underline{C}H_2I$); MS (ES+) *m/z* (relative intensity) 341.0 [MNa^+, 98%], 319.0 [MH^+, 100%].

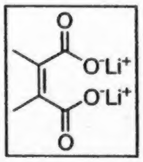
Dimethyl (Z)-2,3-dimethylbutenedioate (33).



The known compound **33**[361-369] was prepared using an adaptation of the method described by Müller.[156] A mixture of dimethylmaleic anhydride (**32**) (8.82 g, 70 mmol), MeOH (98 mL), trimethyl orthoformate (10.2 mL, 92.4 mmol), and *p*-TsOH (1.75 g) was refluxed for 23 h. The mixture was poured into iced-cold H_2O, extracted with CH_2Cl_2, dried (Na_2SO_4), and concentrated *in vacuo*. Further purification by flash chromatography (SiO_2, 1:1/petroleum ether:Et_2O, R_f 0.47) gave **33** (11.18 mg, 93%) as a colorless oil: IR ($CDCl_3$ cast) (lit.[156,361,368]) 3000, 2953, 1723, 1651, 1435, 1269, 1100 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) (lit.[156,361,365,368]) δ 3.74 (s, 6H,

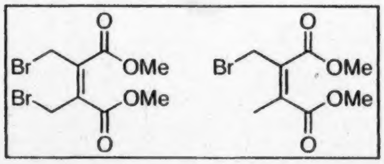
$2xOC\underline{H}_3$), 1.92 (s, 6H, $2xC{=}C\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 169.4 ($2x\underline{C}{=}O$), 133.4 ($\underline{C}{=}\underline{C}$), 52.2 ($2xO\underline{C}H_3$), 15.6 ($2xC{=}\underline{C}H_3$); HRMS (EI) Calcd for $C_8H_{12}O_4$ 172.0736, found 172.0733 $[M]^+$.

***Dimethyl (Z)-2,3-dimethylbutenedioic acid dilithium salt* (34).**



A solution of 1 N LiOH (3.64 mL, 3.64 eq) was added to diester **33** (314 mg, 1.82 mmol) in a $THF{:}H_2O/1{:}1$ solution (4 mL). The reaction mixture was stirred at rt for 1 day. The solvent was removed under reduced pressure and the remaining solid was dissolved in H_2O. Non-polar impurities including unreacted starting material were removed by extraction with Et_2O. Freeze-drying of the aqueous layer gave **34** (275 mg, 97%) as a white solid: IR ($CHCl_3$ cast) 3472, 3382, 2923, 1656, 1593, 1551, 1402, 1226, 1121, 1098, 811, 797, 703 cm^{-1}; ^1H NMR (D_2O, 300 MHz) δ 1.81 (s, 6H, $C\underline{H}_3C{=}C$); ^{13}C NMR (D_2O, 75 MHz) δ 180.5 ($2x\underline{C}{=}O$), 133.7 ($\underline{C}{=}\underline{C}$), 16.2 ($2x\underline{C}H_3$); MS (ES+) *m/z* (relative intensity) 157.1 [MH^+, 100%], 151.0 [$[MH-Li]^+$, 52%].

***Dimethyl (Z)-2,3-di-bromomethylbutenedioate* (35) *and dimethyl (Z)-2-bromomethyl-3-methylbutenedioate* (36).**



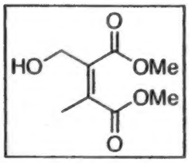
Bromide **36** and the known dibromide **35**[366] were prepared using an adaptation of the method described by Clennan.[370] To a solution of dimethyl (*Z*)-2,3-dimethylbutenedioate (**33**) (325 mg, 1.89 mmol) in CCl_4 (1.2 mL) was added *N*-

bromosuccinimide (331 mg, 1.89 mmol) and dibenzoyl peroxide (1.4 mg). The reaction mixture was stirred at rt for 3 h, and refluxed for 19 h. The solution was then cooled to rt and the succinimide was removed by vacuum filtration and washed with CCl_4. The resulting solution was then concentrated under reduced pressure. Further purification by flash chromatography (SiO_2, 7:3/hexane:EtOAc, R_f**35** 0.36 and R_f**36** 0.39 (2:1/hexane:EtOAc)) gave **35** (175 mg, 28%) and **36** (313, 66%) as colorless oils.

Data for **35**: IR ($CDCl_3$ cast) (lit.[366]) 2952, 1726, 1633, 1434, 1321, 1273, 1214, 1007, 955, 841, 786 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) (lit.[366]) δ 4.22 (s, 4H, 2xBrC$\underline{H}_2$C=C), 3.82 (s, 6H, 2xOC$\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 166.0 (2x$\underline{C}$=O), 137.2 ($\underline{C}$=$\underline{C}$), 53.1 (2xO$\underline{C}H_3$), 24.0 ($\underline{C}H_2$Br); HRMS (EI) Calcd for $C_8H_{10}O_4Br_2$ 329.8926, found 329.8911 $[M]^+$.

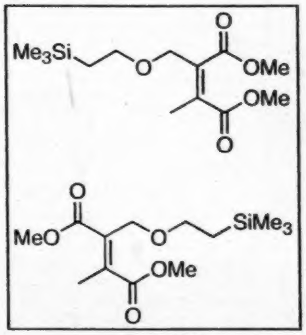
Data for **36**: IR ($CHCl_3$ cast) 2952, 2923, 2852, 1724, 1640, 1435, 1271, 1106, 1044, 943, 763 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 4.18 (s, 2H, BrC$\underline{H}_2$C=C), 3.79 and 3.78 (2s, 6H, 2xOC$\underline{H}_3$), 2.07 (s, 3H, C$\underline{H}_3$C=C); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 169.4 and 165.7 (2x$\underline{C}$=O), 142.5 and 130.1 ($\underline{C}$=$\underline{C}$), 52.7 and 52.6 (2xO$\underline{C}H_3$), 25.2 ($\underline{C}H_2$Br), 16.8 ($\underline{C}H_3$C=C); HRMS (EI) Calcd for $C_8H_{11}O_4$ 171.0657, found 171.0656 $[M-Br]^+$.

***Dimethyl (Z)-2-hydroxymethyl-3-methylbutenedioate* (37).**



This transformation was performed using an adaptation of the method described by Rayner.[371] A mixture of **36** (94 mg, 0.38 mmol), H_2O (1 mL) and K_2CO_3 (68 mg, 0.49 mmol) was heated at 80 °C for 5 h. The cooled reaction mixture was saturated with NaCl, extracted with EtOAc, and concentrated *in vacuo* to give **37** (29 mg, 41%) as a colorless oil: IR ($CHCl_3$ cast) 3425, 2954, 1722, 1651, 1436, 1384, 1270, 1074 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 4.31 (s, 2H, $C\underline{H}_2OH$), 3.77 and 3.76 (2s, 6H, 2x$OC\underline{H}_3$), 2.02 (s, 3H, $C\underline{H}_3C$=C), 1.23 (br s, 1H, $CH_2O\underline{H}$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 169.2 and 167.4 (2x$\underline{C}$=O), 137.9 and 133.7 ($\underline{C}$=$\underline{C}$), 59.0 ($\underline{C}H_2OH$), 52.4 and 52.3 (2x$O\underline{C}H_3$), 16.0 ($\underline{C}H_3C$=C); MS (ES+) *m/z* (relative intensity) 211.1 [MNa^+, 100%], 189.1 [MH^+, 4%].

***Dimethyl (Z)-2-(2'-trimethylsilylethoxy)methyl-3-methylbutenedioate* (38) *and dimethyl (E)-2-(2'-trimethylsilylethoxy)methyl-3-methylbutenedioate* (39).**



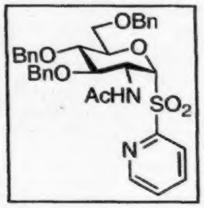
Methylmagnesium chloride (6.7 mL of a 3.0 M solution in THF, 20 mmol) was added dropwise to a suspension of $CuBr{\bullet}Me_2S$ (4.11 g, 20 mmol) in THF (50 mL) at -40 °C. The resulting yellow suspension was stirred at -40 °C for 2 h, then cooled to -78 °C, and freshly distilled DMAD (2.22 mL, 18 mmol) in THF (10 mL) was added dropwise to give a dark red brown mixture. After 1 h, a HMPA:THF/1:1 solution (20 mL) was added dropwise, and the reaction mixture was stirred for 10 min. Trimethylsilylethoxymethyl chloride (7.1 mL, 40 mmol) in THF (10 mL) was then added dropwise and the reaction mixture was stirred for 2 h at -78 °C. The reaction mixture was warmed to -40 °C and stirred for 4.5 h, and then warmed to 0 °C. After 1.5 h, the reaction mixture was quenched with a saturated aq. NH_4Cl solution (50 mL, adjusted to pH 8 with 10% ammonia), and allowed to warm to rt. After 30 min, the mixture was filtered through a pad of Celite®. The aqueous layer was extracted with

EtOAc, and the combined organic extracts were washed with a saturated aq. NH_4Cl solution, H_2O, and brine, dried (Na_2SO_4), and concentrated *in vacuo*. Further purification by flash chromatography (SiO_2, 3:2/petroleum ether:Et_2O, R_f **38** 0.47 (3:2/petroleum ether:Et_2O) and R_f **39** 0.38 (1:4/ petroleum ether:Et_2O)) gave **38** (363 mg, 7%) and **39** (2.54 g, 49%) as colorless oils.

Data for **38**: IR ($CDCl_3$ cast) 2953, 1738, 1719, 1639, 1436, 1302, 1249, 1128, 1084, 858, 837 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 5.03 (s, 2H, $OC\underline{H}_2C{=}C$), 3.81 and 3.71 (2s, 6H, $2xOC\underline{H}_3$), 3.72-3.67 (m, 2H, $Me_3SiCH_2C\underline{H}_2O$), 1.87 (s, 3H, $C\underline{H}_3C{=}C$), 0.94-0.89 (m, 2H, $Me_3SiC\underline{H}_2CH_2O$), 0.00 (s, 9H, $(C\underline{H}_3)_3Si$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 168.1 and 164.6 ($2x\underline{C}{=}O$), 150.9 and 143.5 ($\underline{C}{=}\underline{C}$), 93.9 ($O\underline{C}H_2C{=}C$), 67.1 ($SiCH_2\underline{C}H_2O$), 52.6 and 52.0 ($2xO\underline{C}H_3$), 17.9 ($Si\underline{C}H_2CH_2O$), 11.6 ($\underline{C}H_3C{=}C$), -1.4 ($(\underline{C}H_3)_3Si$); MS (ES+) *m/z* (relative intensity) 327.0 [MNa^+, 100%], 631.2 [$2MNa^+$, 30%].

Data for **39**: IR ($CHCl_3$ cast) 2953, 1730, 1643, 1435, 1247, 1077, 859, 837 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 4.35 (q, 2H, J = 1.2 Hz, $OC\underline{H}_2C{=}C$), 3.78 and 3.76 (2s, 6H, $2xOC\underline{H}_3$), 3.50-3.44 (m, 2H, $Me_3SiCH_2C\underline{H}_2O$), 2.01 (t, 3H, J = 1.2 Hz, $C\underline{H}_3C{=}C$), 0.89-0.84 (m, 2H, $Me_3SiC\underline{H}_2CH_2O$), -0.03 (s, 9H, $(C\underline{H}_3)_3Si$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 168.7 and 168.0 ($2x\underline{C}{=}O$), 137.5 and 133.5 ($\underline{C}{=}\underline{C}$), 68.2 ($O\underline{C}H_2C{=}C$), 67.7 ($SiCH_2\underline{C}H_2O$), 52.1 and 52.0 ($2xO\underline{C}H_3$), 18.1 ($Si\underline{C}H_2CH_2O$), 17.5 ($\underline{C}H_3C{=}C$), -1.4 ($(\underline{C}H_3)_3Si$).

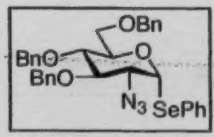
2-Acetamido-3,4,6-tri-O-benzyl-2-deoxy-α-D-glucopyranosyl 2-pyridyl sulfone (40).



The preparation of the known compound **40**[372] was done using the method described by Urban.[173] *m*-CPBA of approximately 85% purity (280 mg, 0.81 mmol) was added to a stirred mixture of α-sulfide **47** (221 mg, 0.38 mmol) and NaHCO$_3$ (223 mg, 2.65 mmol) in CH$_2$Cl$_2$ (4 mL) at 0 °C. Stirring was continued at 0 °C for 2 h, after which time the reaction mixture was diluted with CH$_2$Cl$_2$, washed consecutively with 50% aq. Na$_2$S$_2$O$_3$, a saturated aq. NaHCO$_3$ solution, brine, dried (Na$_2$SO$_4$), and concentrated under reduced pressure. Further purification by flash chromatography (SiO$_2$, 1:5/hexane:EtOAc, R_f 0.47) gave **40** (209 mg, 89%) as a colorless oil: $[\alpha]^{26}_{D}$ +56° (*c* 0.43, CHCl$_3$); IR (CHCl$_3$ cast) 3366, 3062, 3030, 2867, 1665, 1526, 1453, 1121, 1097, 1078, 1028, 749, 698 cm^{-1}; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 8.68 (not well resolved ddd, 1H, SO$_2$C=NC$\underline{H}$), 8.00 (d, 1H, J = 7.8 Hz, SO$_2$C-C$\underline{H}$C=C), 7.81 (td, J = 7.8 Hz, J = 1.5 Hz, SO$_2$C-CHC=C$\underline{H}$), 7.43 (ddd, 1H, J = 7.8 Hz, J = 4.8 Hz, J = 1.2 Hz, SO$_2$C=NCHC=C$\underline{H}$), 7.40-7.18 (m, 15H, 3xC$_6\underline{H}_5$), 5.82 (d, 1H, $J_{NH,2}$ = 8.7 Hz, N$\underline{H}$), 5.52 (d, 1H, $J_{1,2}$ = 5.7 Hz, C$\underline{H}$-1), 4.84 (d, 1H, J = 11.7 Hz, C$\underline{H}$ of CH$_2$C$_6$H$_5$), 4.76 (d, 1H, J = 11.1 Hz, C$\underline{H}$ of CH$_2$C$_6$H$_5$), 4.72 (d, 1H, J = 11.7 Hz, C$\underline{H}$ of CH$_2$C$_6$H$_5$), 4.69 (ddd, 1H, $J_{2,3}$ = 10.5 Hz, $J_{2,NH}$ = 8.7 Hz, $J_{2,1}$ = 5.7 Hz, C$\underline{H}$-2), 4.55 (d, 1H, J = 11.1 Hz, C$\underline{H}$ of CH$_2$C$_6$H$_5$), 4.44 (d, 1H, J = 12.0 Hz, C$\underline{H}$ of CH$_2$C$_6$H$_5$), 4.43-4.34 (m, 2H, C$\underline{H}$-3 and C$\underline{H}$-5), 4.30 (d, 1H, J = 12.0 Hz, C$\underline{H}$ of CH$_2$C$_6$H$_5$), 3.68 (dd, 1H, $J_{4,3}$ = 9.0 Hz, $J_{4,5}$ = 7.8 Hz, C$\underline{H}$-4), 3.61 (dd, 1H, $J_{6,6'}$ = 10.8 Hz, $J_{6,5}$ = 4.5 Hz, C$\underline{H}$-6), 3.47 (dd, 1H, $J_{6',6}$ = 10.8 Hz, $J_{6',5}$ = 2.4 Hz, C$\underline{H}$-6'), 1.77 (s, 3H, C=OC$\underline{H}_3$); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 170.4 ($\underline{C}$=O), 156.3 (S$\underline{C}$=N), 150.3 ($\underline{C}$H=N), 138.0 (SC-C=$\underline{C}$), 137.8 and

137.7 (3x$\underline{C}$-1' of Bn), 128.7, 128.44, 128.38, 128.34, 128.1, 127.9, 127.8, 127.71, 127.69 and 127.6 (3x$\underline{C}_6H_5$ and SC-C=C-$\underline{C}$), 123.5 (SC-$\underline{C}$), 87.4 ($\underline{C}$-1), 77.7 and 76.4 ($\underline{C}$-3 and $\underline{C}$-5), 77.2 ($\underline{C}$-4), 74.8, 74.6 and 73.3 (3x$C_6H_5\underline{C}H_2$-), 68.0 ($\underline{C}$-6), 50.1 ($\underline{C}$-2), 23.1 (NHC=O$\underline{C}H_3$); HRMS (ES+) Calcd for $C_{34}H_{36}N_2O_7SNa$ 639.2135, found 639.2131 $[M+Na]^+$, Calcd for $C_{34}H_{37}N_2O_7S$ 617.2316, found 617.2330 $[M+H]^+$.

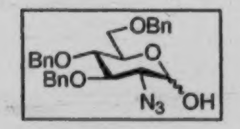
***Phenyl 2-azido-3,4,6-tri-*O*-benzyl-2-deoxy-1-seleno-α-*D*-glucopyranoside* (42).**



The known compound **42**[373,374] was prepared using the procedure described by Czernecki.[175] To a stirred solution of 3,4,6-tri-*O*-benzyl-D-glucal (**41**) (5 g, 12 mmol), azidotrimethylsilane (3.19 mL, 24 mmol), and tetrabutylammonium fluoride (2.4 mL, 2.4 mmol, 1.0 M solution in THF) in CH_2Cl_2 (120 mL) under argon, was added *N*-phenylselenophthalimide (7.25 g, 24 mmol). The mixture was stirred at rt for 2 days. The solvent was evaporated, toluene (180 mL) was added, the precipitated salts were filtered off, and the crude mixture was concentrated. Purification by flash chromatography (SiO_2, 11:1/hexane:Et_2O, R_f 0.45 (4:1/hexane:Et_2O)) gave **42** (3.27 g, 44%) as a white solid: mp 86-88 °C (lit.[175] mp 86-89 °C); $[\alpha]^{26}_{D}$ +149° (*c* 1.18, $CHCl_3$) (lit.[175] $[\alpha]^{22}_{D}$ +99° (*c* 1.00, CH_2Cl_2)) (lit.[374] $[\alpha]^{22}_{D}$ +101° (*c* 1.00, $CHCl_3$)); IR ($CHCl_3$ cast) (lit.[175,374]) 3030, 2865, 2107, 1577, 1495, 1453, 1361, 1084, 1070, 1027, 735, 696 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) (lit.[175,374]) δ 7.59-7.13 (m, 20H, 4x$C_6\underline{H}_5$), 5.88 (d, 1H, $J_{1,2}$ = 5.1 Hz, C$\underline{H}$-1), 4.88-4.38 (m, 6H, 3x$C_6H_5C\underline{H}_2$-), 4.27-4.19 (m, 1H, C$\underline{H}$-5), 3.88 (dd, 1H, $J_{2,3}$ = 10.2 Hz, $J_{2,1}$ = 5.1 Hz, C$\underline{H}$-2), 3.77 (dd, $J_{4,5}$ = 7.5 Hz, $J_{4,3}$ = 3.6 Hz, C$\underline{H}$-4), 3.76-3.68 (m, 2H, C$\underline{H}$-3 and C$\underline{H}$-6), 3.54 (dd, 1H, $J_{6',6}$ = 10.8 Hz, $J_{6',5}$ = 2.1 Hz, C$\underline{H}$-6');

^{13}C NMR ($CDCl_3$, 75 MHz) δ 137.9, 137.8 and 137.7 (3x$\underline{C}$-1' of Bn), 134.6, 129.2, 128.6, 128.5, 128.4, 128.2, 128.0, 127.9, 127.83 and 127.79 (4x$\underline{C}_6H_5$), 85.3 ($\underline{C}$-1), 82.8 ($\underline{C}$-3), 78.0 ($\underline{C}$-4), 75.8, 75.2 and 73.53 (3x$C_6H_5\underline{C}H_2$-), 73.50 ($\underline{C}$-5), 68.2 ($\underline{C}$-6), 64.8 ($\underline{C}$-2); HRMS (ES+) Calcd for $C_{33}H_{33}N_3O_4NaSe$ 638.5912, found 638.1539 $[M+Na]^+$.

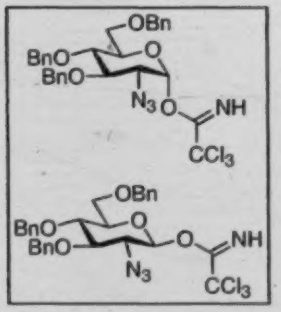
***2-Azido-3,4,6-tri-O-benzyl-2-deoxy-D-glucopyranose* (43).**



The known compound **43**[174,373,375-377] was prepared using the method described by Czernecki.[175] A solution of **42** (3.263 g, 5.31 mmol) in a THF:H_2O/1:1 solution (10.6 mL) was treated at rt with mercury trifluoroacetate (3.397 g, 7.97 mmol). After 1 h, the reaction mixture was diluted with EtOAc (55 mL), and washed with a saturated aq. K_2CO_3 solution (55 mL). The aqueous layer was extracted with EtOAc (2x30 mL). The combined organic layers were washed with Na_2S (55 mL). The aqueous layer was re-extracted with EtOAc (2x55 mL). The combined organic layers were washed until obtention of a neutral pH, dried ($MgSO_4$), filtered, and concentrated under reduced pressure to provide a crude orange oil. Further purification by flash chromatography (SiO_2, 1:2/hexane:Et_2O, R_f 0.69) gave **43** (1,338 g, 53%) as a white solid: mp 98-100 °C (lit.[174] mp 96-100 °C); IR ($CHCl_3$ cast) 3406, 3067, 3017, 2924, 2111, 1496, 1455, 1361, 1216, 1122, 1054, 1028, 755, 699, 668 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) (lit.[174,376,377]) δ 7.38-7.10 (m, 15H, 3x$C_6\underline{H}_5$), 5.32 (t, 1H, J = 3.3 Hz, C$\underline{H}$-1α), 4.89-4.47 (m, 7H, 3x$C_6H_5C\underline{H}_2$- and C$\underline{H}$-1β), 4.14-3.96 (m, 3H, C$\underline{H}$-3α, C$\underline{H}$-5α and C$\underline{H}$-5β), 3.74-3.55 (m, 5H, C$\underline{H}$-4α, C$\underline{H}_2$-6α and C$\underline{H}_2$-6β), 3.50-3.32 (m, 4H, C$\underline{H}$-2α, C$\underline{H}$-2β, C$\underline{H}$-3β and C$\underline{H}$-4β) 3.19 and 2.75 (2xbr s, 2x1H, 2xO$\underline{H}$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ

137.94, 137.89, 137.8 and 137.7 (4x$\underline{C}$-1' of Bn), 128.55, 128.53, 128.49, 128.45, 128.2, 128.1, 128.00, 127.95, 127.85 and 127.83 (3x$\underline{C}_6H_5$), 96.3 ($\underline{C}$-1β), 92.2 ($\underline{C}$-1α), 83.1 ($\underline{C}$-3β), 80.2 ($\underline{C}$-3α), 78.5 ($\underline{C}$-4α), 77.7 ($\underline{C}$-4β), 75.63, 75.59, 75.10, 75.06, 73.65 and 73.59 (3x$C_6H_5\underline{C}H_2$-), 72.3 ($\underline{C}$-5β), 70.8 ($\underline{C}$-5α), 68.7 ($\underline{C}$-6β), 68.6 ($\underline{C}$-6α), 67.6 ($\underline{C}$-2β), 64.1 ($\underline{C}$-2α); HRMS (ES+) Calcd for $C_{27}H_{29}N_3O_5Na$ 498.2005, found 498.2002 $[M+Na]^+$.

***2-Azido-3,4,6-tri-O-benzyl-2-deoxy-α-D-glucopyranosyltrichloroacetimidate* (44) *and 2-azido-3,4,6-tri-O-benzyl-2-deoxy-β-D-glucopyranosyltrichloroacetimidate* (45).**



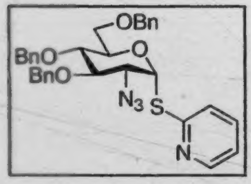
The known compounds **44** and **45**[378] were prepared using an imidation method described by Urban.[173] To a stirred solution of **43** (43 mg, 0.09 mmol) and trichloroacetonitrile (54 μL, 0.54 mmol) in dry CH_2Cl_2 (1 mL) was added K_2CO_3 (19 mg, 0.14 mmol). The mixture was stirred at 30 °C for 17 h, after which it was filtered and evaporated to dryness *in vacuo*. Further purification by flash chromatography (SiO_2, 5:1/hexane:EtOAc containing 1% Et_3N, R_f **44** 0.38 and R_f **45** 0.31) gave **44** (2 mg, 4%) as a colorless oil and **45** (41 mg, 74%) as a white solid.

Data for **44**: $[\alpha]_D^{26}$ +52° (*c* 1.66, $CHCl_3$); IR ($CHCl_3$ cast) 3338, 3088, 3064, 3031, 2922, 2867, 2111, 1673, 1497, 1454, 1384, 1362, 1274, 1209, 1144, 1072, 1027, 967, 915, 857, 837, 796, 736, 698 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 8.69 (s, 1H, N$\underline{H}$), 7.37-7.14 (m, 15H, 3x$C_6\underline{H}_5$), 6.42 (d, 1H, $J_{1,2}$ = 3.6 Hz, C$\underline{H}$-1), 4.94-4.44 (2m, 6H, 3x$C_6H_5C\underline{H}_2$-), 4.05-3.83 (m, 3H, C$\underline{H}$-3, C$\underline{H}$-4 and C$\underline{H}$-5), 3.79 (dd, 1H, $J_{6,6'}$ = 11.1 Hz, $J_{6,5}$ = 2.7 Hz, C$\underline{H}$-6), 3.68 (dd, 1H, $J_{2,3}$ = 10.2 Hz, $J_{2,1}$ = 3.6 Hz, C$\underline{H}$-2), 3.65 (dd, 1H, $J_{6',6}$ = 11.1 Hz, $J_{6',5}$ = 1.8

Hz, C$\underline{H}$-6'); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 160.9 ($\underline{C}$=NH), 137.8, 137.7 and 137.6 (3x$\underline{C}$-1' of Bn), 130.6, 128.5, 128.4, 128.1, 128.00, 127.96 and 127.8 (3x$\underline{C}_6$H$_5$), 95.0 ($\underline{C}$-1), 83.8 ($\underline{C}$Cl$_3$), 80.1, 77.7 and 77.62 ($\underline{C}$-3, $\underline{C}$-4 and $\underline{C}$-5), 75.6, 75.3 and 73.61 (3xC$_6$H$_5\underline{C}$H$_2$-), 67.9 ($\underline{C}$-6), 63.2 ($\underline{C}$-2); HRMS (ES+) Calcd for $C_{29}H_{29}N_4O_5NaCl_3$ 641.1096, found 641.1098 [M+Na]$^+$.

Data for **45**: mp 102-104 °C (lit.[378] mp 103-104 °C); $[\alpha]^{26}_D$ -14° (*c* 0.25, CHCl$_3$) (lit.[378] $[\alpha]^{26}_D$ -3° (*c* 1.15, CHCl$_3$)); IR (CDCl$_3$ cast) 3337, 3064, 3031, 2869, 2113, 1676, 1496, 1454, 1359, 1286, 1061, 1029, 835, 797, 697 cm^{-1}; ^{1}H NMR (CDCl$_3$, 300 MHz) (lit.[378]) δ 8.73 (s, 1H, N$\underline{H}$), 7.36-7.16 (m, 15H, 3xC$_6\underline{H}_5$), 5.62 (d, 1H, $J_{1,2}$ = 8.4 Hz, C$\underline{H}$-1), 4.92-4.50 (2m, 6H, 3xC$_6$H$_5$C$\underline{H}_2$-), 3.80-3.51 (m, 5H, C$\underline{H}$-3, C$\underline{H}$-4, C$\underline{H}$-5 and C$\underline{H}_2$-6), 3.75 (d, 1H, $J_{2,1}$ = 8.4 Hz, C$\underline{H}$-2); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 161.1 ($\underline{C}$=NH), 138.0, 137.85 and 137.83 (3x$\underline{C}$-1' of Bn), 128.52, 128.49, 128.4, 128.1, 128.0, 127.92, 127.89 and 127.7 (3x$\underline{C}_6$H$_5$), 96.9 ($\underline{C}$-1), 90.6 ($\underline{C}$Cl$_3$), 83.1, 77.3 and 76.1 ($\underline{C}$-3, $\underline{C}$-4 and $\underline{C}$-5), 75.7, 75.1 and 73.5 (3xC$_6$H$_5\underline{C}$H$_2$-), 68.0 ($\underline{C}$-6), 65.9 ($\underline{C}$-2); MS (ES+) *m/z* (relative intensity) 641.1 [MNa$^+$, 55%]; Anal. Calcd for $C_{29}H_{29}Cl_3N_4O_5$: C, 56.19; H, 4.72; N, 9.04. Found: C, 55.86; H, 4.64; N, 8.85.

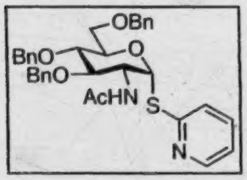
***2-Pyridyl 2-azido-3,4,6-tri-*O*-benzyl-2-deoxy-1-thio-α-*D*-glucopyranoside* (46).**



The known compound **46**[372] was prepared using a method described by Urban.[173] β-imidate **45** (642 mg, 1.04 mmol) was dissolved in CH$_2$Cl$_2$ (34 mL), and 2-mercaptopyridine (16 mg, 0.14 mmol) was added. The stirred solution was cooled to -15 °C, and BF$_3$•Et$_2$O (34 μL, 0.25

mmol) was added. After being stirred at this temperature for 19 h, the reaction mixture was washed with a saturated aq. $NaHCO_3$ solution, dried (Na_2SO_4), and concentrated under reduced pressure. Further purification by flash chromatography (SiO_2, 5:2/hexane:EtOAc, R_f 0.40) gave **46** (517 mg, 88%) as a white solid: mp 76-77 °C; $[\alpha]_D^{26}$ +123° (*c* 0.14, $CHCl_3$); IR ($CDCl_3$ cast) 3030, 2923, 2868, 2108, 1577, 1560, 1496, 1453, 1147, 1089, 1052, 1027, 736, 697 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 8.47 (ddd, 1H, J_{ortho} = 4.8 Hz, J_{meta} = 1.8 Hz, J_{para} = 0.9 Hz, $C\underline{H}$=N), 7.56-7.04 (3m, 18H, $3xC_6\underline{H}_5$ and 3$\underline{H}$-pyr), 6.59 (d, 1H, $J_{1,2}$ = 5.4 Hz, $C\underline{H}$-1), 4.90 (d, 1H, J = 10.5 Hz, $C\underline{H}$ of $CH_2C_6H_5$), 4.85 (d, 1H, J = 10.8 Hz, $C\underline{H}$ of $CH_2C_6H_5$), 4.80 (d, 1H, J = 10.8 Hz, $C\underline{H}$ of $CH_2C_6H_5$), 4.53 (d, 2H, J = 11.7 Hz, $C\underline{H}_2C_6H_5$), 4.38 (d, 1H, J = 11.7 Hz, $C\underline{H}$ of $CH_2C_6H_5$), 4.15-4.07 (m, 1H, $C\underline{H}$-5), 4.01 (dd, $J_{2,3}$ = 10.2 Hz, $J_{2,1}$ = 5.4 Hz, $C\underline{H}$-2), 3.77-3.71 (m, 3H, $C\underline{H}$-3, $C\underline{H}$-4 and $C\underline{H}$-6), 3.58 (dd, 1H, $J_{6',6}$ = 11.1 Hz, $J_{6',5}$ = 1.8 Hz, $C\underline{H}$-6'); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 155.7 ($S\underline{C}$=N), 149.4 ($\underline{C}H$=N), 137.93, 137.89 and 137.7 (3x$\underline{C}$-1' of Bn), 137.0 (SC-C=$\underline{C}$), 128.54, 128.50, 128.4, 128.2, 128.0, 127.9, 127.8 and 127.7 ($3x\underline{C}_6H_5$), 124.2 (SC-$\underline{C}$), 120.9 (SC-C=C-$\underline{C}$), 83.5 ($\underline{C}$-1), 82.4 and 78.1 ($\underline{C}$-3 and $\underline{C}$-4), 75.8, 75.1 and 73.4 ($3xC_6H_5\underline{C}H_2$-), 73.5 ($\underline{C}$-5), 68.3 ($\underline{C}$-6), 64.0 ($\underline{C}$-2); MS (ES+) *m/z* (relative intensity) 591.1 [MNa^+, 100%], 569.2 [MH^+, 74%]; Anal. Calcd for $C_{32}H_{32}N_4O_4S$: C, 67.58; H, 5.67; N, 9.85. Found: C, 67.14; H, 5.57; N, 9.94.

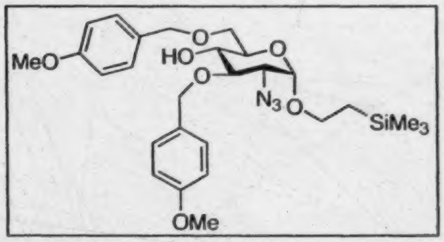
2-Pyridyl 2-acetamido-3,4,6-tri-O-benzyl-2-deoxy-1-thio-α-D-glucopyranoside (47).



The preparation of the known compound **47**[372] was performed using the method described by Urban.[173] To a stirred solution of $SnCl_2$ (162 mg, 0.85 mmol) in dry MeCN (4.8 mL) was added consecutively thiophenol (352 μL, 3.43 mmol), Et_3N (356 μL, 2.57 mmol), and azide **46** (325 mg, 0.57 mmol). The reaction mixture was stirred at rt for 35 min, after which time it was diluted with CH_2Cl_2, and washed with 2 N NaOH. The aqueous layer was re-extracted with CH_2Cl_2, and the combined organic layers were dried (Na_2SO_4), and evaporated to dryness *in vacuo*. The residue was treated with pyridine (8.6 mL) and acetic anhydride (3.3 mL) at rt overnight. The reaction mixture was evaporated to dryness *in vacuo* and then coevaporated thrice with toluene. Further purification by flash chromatography (SiO_2, 1:3/hexane:EtOAc, R_f 0.21) gave **47** (330 mg, 99%) as a white solid: mp 150-151 °C; $[\alpha]_D^{26}$ +171° (*c* 0.17, $CHCl_3$); IR ($CHCl_3$ cast) 3317, 3030, 2923, 2856, 1653, 1537, 1452, 1121, 1090, 1050, 1028, 741, 696 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 8.39 (not well resolved ddd, 1H, C$\underline{H}$=N), 7.51-7.00 (3m, 18H, 3x$C_6\underline{H}_5$ and 3$\underline{H}$-pyr), 6.31 (d, 1H, $J_{1,2}$ = 4.8 Hz, C$\underline{H}$-1), 5.27 (d, 1H, $J_{NH,2}$ = 8.4 Hz, N$\underline{H}$), 4.85 (d, 1H, J = 12.3 Hz, C$\underline{H}$ of $CH_2C_6H_5$), 4.81 (d, 1H, J = 11.4 Hz, C$\underline{H}$ of $CH_2C_6H_5$), 4.66 (d, 1H, J = 11.7 Hz, C$\underline{H}$ of $CH_2C_6H_5$), 4.60 (d, 1H, J = 12.3 Hz, C$\underline{H}$ of $CH_2C_6H_5$), 4.58 (d, 1H, J = 11.1 Hz, C$\underline{H}$ of $CH_2C_6H_5$), 4.52 (ddd, 1H, $J_{2,3}$ = 10.5 Hz, $J_{2,NH}$ = 8.4 Hz, $J_{2,1}$ = 4.8 Hz, C$\underline{H}$-2), 4.46 (d, 1H, J = 12.3 Hz, C$\underline{H}$ of $CH_2C_6H_5$), 4.15 (ddd, 1H, $J_{5,4}$ = 9.3 Hz, $J_{5,6}$ = 3.6 Hz, $J_{5,6'}$ = 2.1 Hz, C$\underline{H}$-5), 3.85-3.79 (m, 2H, C$\underline{H}$-4 and C$\underline{H}$-6), 3.69 (d, 1H, $J_{6',5}$ = 2.1 Hz, C$\underline{H}$-6'), 3.62 (dd, 1H, $J_{3,2}$ = 10.5 Hz, $J_{3,4}$ = 8.4 Hz, C$\underline{H}$-3), 1.74 (s, 3H, C=OC$\underline{H}_3$); ^{13}C NMR

($CDCl_3$, 75 MHz) δ 169.9 (C=O), 156.7 (SC=N), 149.5 (CH=N), 138.05, 137.95 and 137.88 (3xC-1' of Bn), 136.9 (SC-C=C), 128.7, 128.44, 128.41, 128.3, 128.1, 128.0, 127.9, 127.8 and 127.6 (3xC_6H_5), 123.5 (SC-C), 120.8 (SC-C=C-C), 84.6 (C-1), 79.8 (C-3), 78.1 (C-4), 74.9, 74.5 and 73.3 (3x$C_6H_5CH_2$-), 73.8 (C-5), 68.4 (C-6), 52.4 (C-2), 23.3 (NHC=OCH_3); HRMS (ES+) Calcd for $C_{34}H_{36}N_2O_5SNa$ 607.2243, found 607.2250 $[M+Na]^+$.

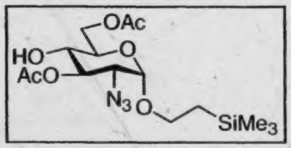
***(2-Trimethylsilyl)ethyl 2-azido-2-deoxy-3,6-di-*O*-(p-methoxybenzyl)-α-*D*-glucopyranoside* (50).**



This transformation was based on a combination of the methods described by Grandjean,[379] Johansson,[380] and Chowdhury.[381] A solution of TFA (3.37 mL, 32.8 mmol) in DMF (15 mL), precooled at 0 °C, was added dropwise to a stirred mixture containing **59** (1.19 g, 2.19 mmol), sodium cyanoborohydride (1.36 g, 21.9 mmol), and 4 Å molecular sieves in DMF (16 mL). The reaction mixture was stirred at rt for 23 h. The reaction mixture was then filtered through Celite®. The filtrate was poured onto ice-cold saturated aq. $NaHCO_3$. The aqueous layer was extracted with CH_2Cl_2. The combined organic extracts were washed with brine, dried (Na_2SO_4), filtered, and concentrated under reduced pressure. The residue obtained was purified by flash chromatography (SiO_2; 3:2/hexane:EtOAc, R_f 0.43) to give **50** (889 mg, 75%) as a white solid: mp 58-59 °C; $[\alpha]_D^{26}$ +40° (*c* 1.11, $CHCl_3$); IR ($CHCl_3$ cast) 3480, 2952, 2836, 2105, 1613, 1514, 1464, 1248, 1174, 1111, 1036, 837 cm^{-1}; ^1H

NMR ($CDCl_3$, 300 MHz) δ 7.31 (dt, 2H, J = 5.7 Hz, J = 1.8 Hz, CH-2 of PMB), 7.22 (dt, 2H, J = 5.7 Hz, J = 1.8 Hz, CH-2 of PMB), 6.87 (dt, 2H, J = 5.7 Hz, J = 1.8 Hz, CH-3 of PMB), 6.85 (dt, 2H, J = 5.7 Hz, J = 1.8 Hz, CH-3 of PMB), 4.88 (d, 1H, $J_{1,2}$ = 3.5 Hz, CH-1), 4.83 (d, 1H, $J_{4',4''}$ = 11.0 Hz, CH-4' part of $OCH_2C_6H_5$), 4.69 (d, 1H, $J_{4'',4'}$ = 11.0 Hz, CH-4" part of $OCH_2C_6H_5$), 4.51 (d, 1H, $J_{4',4''}$ = 12.0 Hz, CH-4' part of $OCH_2C_6H_5$), 4.45 (d, 1H, $J_{4'',4'}$ = 12.0 Hz, CH-4" part of $OCH_2C_6H_5$), 3.78 (ddd, 1H, $J_{5,4}$ = 10.2 Hz, $J_{5,6}$ = 4.8 Hz, $J_{5,6'}$ = 2.4 Hz, CH-5), 3.78 (s, 6H, 2xOCH_3), 3.78 (dd, 1H, $J_{3,4}$ = 11.1 Hz, $J_{3,2}$ = 10.5 Hz, CH-3), 3.76 (ddd, 1H, $J_{1',1''}$ = 11.5 Hz, $J_{1',2'}$ = 10.0 Hz, $J_{1',2''}$ = 6.6 Hz, CH-1'), 3.64 (t, 1H, $J_{4,3}$ = 11.1 Hz, CH-4), 3.64 (dd, 1H, $J_{6,6'}$ = 13.5 Hz, $J_{6,5}$ = 4.8 Hz, CH-6), 3.63 (d, 1H, $J_{6',6}$ = 13.5 Hz, $J_{6',5}$ = 2.4 Hz, CH-6'), 3.53 (ddd, 1H, $J_{1'',1'}$ = 11.5 Hz, $J_{1'',2''}$ = 10.0 Hz, $J_{1'',2'}$ = 6.6 Hz, CH-1"), 3.27 (dd, 1H, $J_{2,3}$ = 10.5 Hz, $J_{2,1}$ = 3.5 Hz, CH-2), 1.62 (br s, 1H, OH), 1.06-0.92 (m, 2H, $CH_2Si(CH_3)_3$), 0.01 (s, 9H, $Si(CH_3)_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 159.7 and 159.6 (2xC-1 of PMB), 130.6 and 130.12 (2xC-4 of PMB), 130.06 and 129.6 (2xC-2 of PMB), 114.3 and 114.1 (2xC-3 of PMB), 97.5 (C-1), 79.8 (C-5), 74.9 and 73.6 (2x$OCH_2C_6H_5$), 72.5 (C-4), 70.3 (C-3), 69.8 (C-6), 66.0 (C-1'), 63.1 (C-2), 55.52 and 55.51 (2xOCH_3), 18.3 ($CH_2Si(CH_3)_3$), -1.2 ($Si(CH_3)_3$); HRMS (ES+) Calcd for $C_{27}H_{39}N_3O_7SiNa$ 568.2455, found 568.2459 $[M+Na]^+$.

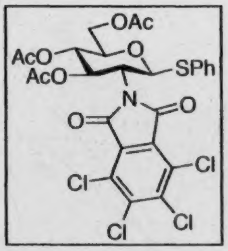
***(2-Trimethylsilyl)ethyl 2-azido-2-deoxy-3,6-di-O-acetyl-α-D-glucopyranoside* (51).**



This deprotection was done using an adaptation of the method described by Miyashita.[382] A solution of a mixture of **69** and **70** (1.703 g, 4.41 mmol) and PPTS (111 mg, 0.44 mmol) in

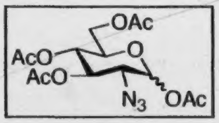
MeOH (40 mL) was stirred at 55 °C for 10 h. The solvent was evaporated under reduced pressure. Further purification by flash chromatography (SiO_2; 2:1/hexane:EtOAc, R_f 0.19) gave **51** (458 mg, 34%) as a colorless oil: $[\alpha]_D^{26}$ +108° (*c* 0.47, $CHCl_3$); IR ($CHCl_3$ cast) 3468, 2954, 2924, 2108, 1747, 1371, 1250, 1228, 1141, 1033, 861, 838 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 5.28 (dd, 1H, $J_{3,2}$ = 10.5 Hz, $J_{3,4}$ = 9.0 Hz, CH-3), 4.94 (d, 1H, $J_{1,2}$ = 3.6 Hz, CH-1), 4.34 (dd, 1H, $J_{6,6'}$ = 12.3 Hz, $J_{6,5}$ = 4.8 Hz, CH-6), 4.27 (dd, 1H, $J_{6',6}$ = 12.3 Hz, $J_{6',5}$ = 2.1 Hz, CH-6'), 3.86 (ddd, 1H, $J_{5,4}$ = 9.9 Hz, $J_{5,6}$ = 4.8 Hz, $J_{5,6'}$ = 2.1 Hz, CH-5), 3.78 (ddd, 1H, $J_{1',1''}$ = 10.2 Hz, $J_{1',2'}$ = 9.6 Hz, $J_{1',2''}$ = 6.3 Hz, CH-1'), 3.56 (ddd, 1H, $J_{1'',1'}$ = 11.1 Hz, $J_{1'',2''}$ = 9.6 Hz, $J_{1'',2'}$ = 6.3 Hz, CH-1"), 3.84 (dt, 1H, $J_{4,5}$ = 9.9 Hz, J = 4.8 Hz, CH-4), 3.24 (dd, 1H, $J_{2,3}$ = 10.5 Hz, $J_{2,1}$ = 3.6 Hz, CH-2), 2.90-2.85 (m, 1H OH), 2.15 and 2.09 (2s, 6H, 2xC=OCH$_3$), 1.00 (2 overlapping ddd, 2H, J = 13.8 Hz, J = 11.1 Hz, J = 6.3 Hz, $CH_2Si(CH_3)_3$), 0.02 (s, 9H, $Si(CH_3)_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 171.8 and 171.4 (2xC=O), 97.2 (C-1), 73.6 (C-3), 70.2 (C-5), 69.7 (C-4), 66.3 (C-1'), 63.1 (C-6), 60.8 (C-2), 21.0 and 20.9 (2xC=OCH$_3$), 18.1 ($CH_2Si(CH_3)_3$), -1.4 ($Si(CH_3)_3$); HRMS (ES+) Calcd for $C_{15}H_{27}N_3O_7SiNa$ 412.1516, found 412.1514 $[M+Na]^+$.

***Phenyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-tetrachlorophthalimido-1-thio-β-*D*-glucopyranoside* (52).**



The known compound **52**[383] was prepared using the method described by Deng.[384] To a solution of a mixture of **60** and **61** (276 mg, 0.45 mmol) in dry CH_2Cl_2 (2 mL) was added thiophenol (60 μL, 0.59 mmol) and BF_3•Et_2O (0.56 mL, 4.39 mmol). After being stirred at rt under argon for 17 h, the reaction was quenched with a saturated aq. $NaHCO_3$ solution (4 mL), then extracted with CH_2Cl_2. The organic layers were washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure. The residue obtained was purified by flash chromatography (SiO_2; 2:1/hexane:EtOAc, R_f 0.29) to give **52** (279 mg, 93%) as a white solid: mp 181-183 °C (lit.[383] mp 181-183 °C); $[\alpha]_D^{26}$ +53° (*c* 0.63, $CHCl_3$) (lit.[383] $[\alpha]_D^{26}$ +55.6° (*c* 1, $CHCl_3$)); IR ($CHCl_3$ cast) 1782, 1749, 1726, 1481, 1440, 1371, 1354, 1301, 1228, 1078, 1044, 914, 752, 741 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) (lit.[383]) δ 7.40-7-24 (m, 5H, C_6H_5), 5.68 (dd, 1H, $J_{3,2}$ = 9.9 Hz, $J_{3,4}$ = 9.0 Hz, CH-3), 5.64 (d, 1H, $J_{1,2}$ = 10.5 Hz, CH-1), 5.11 (dd, 1H, $J_{4,5}$ = 10.2 Hz, $J_{4,3}$ = 9.0 Hz, CH-4), 4.30 (t, 1H, $J_{2,1}$ = 10.5 Hz, CH-2), 4.26 (dd, 1H, $J_{6,6'}$ = 12.3 Hz, $J_{6,5}$ = 5.1 Hz, CH-6), 4.13 (dd, 1H, $J_{6',6}$ = 12.3 Hz, $J_{6',5}$ = 2.4 Hz, CH-6'), 3.83 (ddd, $J_{5,4}$ = 10.2 Hz, $J_{5,6}$ = 5.1 Hz, $J_{5,6'}$ = 2.4 Hz, CH-5), 2.07, 2.00 and 1.84 (3s, 9H, 3xC=OCH$_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 170.5, 169.3, 163.3 and 162.2 (4xC=O), 140.8, 140.6, 133.3, 130.5, 130.1, 129.0, 128.6, 127.1 and 126.8 (C=C of SPh and TCP), 82.4 (C-1), 76.0 (C-5), 71.7 (C-3), 68.4 (C-4), 62.1 (C-6), 54.4 (C-2), 20.8, 20.6 and 20.5 (3xC=OCH$_3$); HRMS (ES+) Calcd for $C_{26}H_{21}Cl_4NO_9SNa$ 685.9589, found 685.9599 [M+Na]$^+$.

***1,3,4,6-Tetra-O-acetyl-2-azido-2-deoxy-D-glucopyranoside* (54).**



The known compound **54** α,[385-387] β,[385,388,389] and α:β mixture was prepared as a α:β mixture using the method described by Vasella.[207]

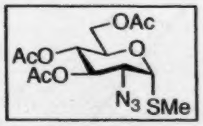
Preparation of Triflic azide: Sodium azide (5.46 g, 84 mmol) was dissolved in H_2O (13.7 mL) under argon at rt. CH_2Cl_2 (17.1 mL) was added to the vigorously stirred solution at 0

°C. Triflic anhydride (freshly distilled over P_2O_5 under argon, 2.4 mL, 14.6 mmol) was added within 30 min. The mixture was stirred for 2 h at 0 °C, the organic layer was separated, and the aqueous layer extracted with CH_2Cl_2 (2x6 mL). The combined organic layers were washed with a saturated aq. $NaHCO_3$ solution (13.7 mL), and H_2O (13.7 mL), dried ($MgSO_4$), filtered, and the triflic azide solution thus obtained was stored at 4 °C over 4 Å molecular sieves (*c* 0.4 M).

***Preparation of* 54:** A suspension of *D*-glucosamine hydrochloride (**53**) (1 g, 4.6 mmol) in MeOH (20 mL) was treated with a 0.5 M solution of sodium methoxide in MeOH (11 mL, 5.5 mmol), and stirred for 10 min at rt. Dilution with MeOH (49 mL), and treatment with 4-(dimethylamino)pyridine (600 mg, 4.9 mmol) furnished a clear colorless solution to which the 0.4 M TfN_3 solution (30 mL, 12 mmol) was added at rt within 10 min by syringe. After stirring at rt under argon for 19 h, the solvent was evaporated *in vacuo*. The residue in pyridine (30 mL) was treated at 0 °C with acetic anhydride (20 mL) and stirred overnight under argon at this temperature. It was diluted with CH_2Cl_2 (250 mL) and washed with 1 M aq. HCl (2x250 mL). The combined aqueous layers were extracted with CH_2Cl_2 (3x100 mL). The combined organic layers were washed with a saturated aq. $NaHCO_3$ solution, and brine, dried (Na_2SO_4), and concentrated under reduced pressure. Further purification by flash chromatography (SiO_2, 2:1/hexane:EtOAc, R_f 0.22) gave **54** (1.31 g, 76%) as a white solid: $[\alpha]_D^{26}$ +51° (*c* 0.8, $CHCl_3$) (lit.[207] $[\alpha]_D^{26}$ +51.6° (*c* 0.8, $CDCl_3$)); IR ($CDCl_3$ cast) 2114, 1755, 1368, 1215, 1075, 1045 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) (α-D:β-D/2:3) δ 6.27 (d, 0.4H, $J_{1,2}$ = 3.6 Hz, $C\underline{H}$-1), 5.53 (d, 0.6H, $J_{1,2}$ = 8.4 Hz, $C\underline{H}$-1), 5.43 (dd, 0.4H, $J_{3,2}$ = 10.5 Hz, $J_{3,4}$ = 9.6 Hz, $C\underline{H}$-3), 5.12-4.98 (m, 1.6H, $C\underline{H}$-3 and 2x$C\underline{H}$-4), 4.28 (dd, 0.6H, $J_{6,6'}$ = 12.6 Hz, $J_{6,5}$ = 4.5 Hz, $C\underline{H}$-6), 4.27 (dd, 0.4H, $J_{6,6'}$ =

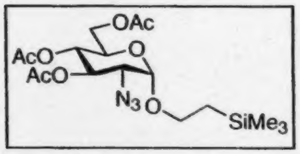
12.3 Hz, $J_{6,5}$ = 3.9 Hz, CH-6), 4.02 (dd, 0.6H, $J_{6',6}$ = 12.6 Hz, $J_{6',5}$ = 2.2 Hz, CH-6'), 4.08-4.00 (m, 0.8H, CH-5 and CH-6'), 3.78 (ddd, 0.6H, $J_{5,4}$ = 9.6 Hz, $J_{5,6}$ = 4.5 Hz, $J_{5,6'}$ = 2.2 Hz, CH-5), 3.64 (dd, 0.4H, $J_{2,3}$= 10.5 Hz, $J_{2,1}$ = 3.6 Hz, CH-2), 3.64 (dd, 0.6H, $J_{2,3}$= 9.8 Hz, $J_{2,1}$ = 8.4 Hz, CH-2), 2.17, 2.16, 2.08, 2.07, 2.05, 2.02 and 2.00 (7s, 8H, 8xC=OCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 170.5, 170.0, 169.8, 169.6, 169.5, 168.54 and 168.49 (C=O), 92.6, 90.0, 72.8, 72.7, 70.8, 69.8, 67.9, 67.8, 62.6, 61.4, 60.3, 20.92, 20.88, 20.69, 20.67, 20.62, 20.55; MS (ES+) Calcd for $C_{14}H_{19}N_3O_9$ 373.11, found 396.1 $[M+Na]^+$.

***Methyl 3,4,6-tri-O-acetyl-2-azido-2-deoxy-1-thio-α-D-glucopyranoside* (55).**



The known compound **55**[181] was prepared using the method described by Pozsgay.[181] A solution of **54** (1.05 g, 2.82 mmol), (methylthio)trimethylsilane (1.05 mL, 7.40 mmol), and TMSOTf (0.09 mL, 0.50 mmol) in anhydrous $(CH_2Cl)_2$ (4.2 mL) was stirred under reflux for 14 h. The solution was concentrated to a syrup. A solution of the residue in $CHCl_3$ (20 mL) was extracted with cold, 5% aq. $NaHCO_3$, H_2O, dried (Na_2SO_4) and concentrated *in vacuo*. Crystallization of the residue from EtOH gave **55** (662 mg, 65%) as a white solid: R_f 0.25 (2:1/hexane:EtOAc) mp 95-97 °C (lit.[181] mp 96-98 °C); $[\alpha]_D^{26}$ +155° (*c* 0.15, $CHCl_3$) (lit.[181] $[\alpha]_D^{22}$ +167° (*c* 0.91, $CHCl_3$)); IR ($CHCl_3$ cast) 2924, 2110, 1748, 1367, 1227, 1087, 1053 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) (lit.[181]) δ 5.31 (d, 1H, $J_{1,2}$ = 5.4 Hz, CH-1), 5.28 (dd, 1H, $J_{3,2}$ = 10.5 Hz, $J_{3,4}$ = 9.3 Hz, CH-3), 4.99 (dd, 1H, $J_{4,5}$ = 9.9 Hz, $J_{4,3}$ = 9.3 Hz, CH-4), 4.37 (ddd, 1H, $J_{5,4}$ = 9.9 Hz, $J_{5,6}$ = 4.8 Hz, $J_{5,6'}$ = 2.1 Hz, CH-5), 4.28 (dd, 1H, $J_{6,6'}$ = 12.3 Hz, $J_{6,5}$ = 4.8 Hz, CH-6), 4.06 (dd, 1H, $J_{6',6}$ = 12.3 Hz, $J_{6',5}$ = 2.1 Hz, CH-6), 4.00 (dd, 1H, $J_{2,3}$ = 10.5 Hz, $J_{2,1}$ = 5.4 Hz, CH-2), 2.10 (s, 3H, SCH$_3$), 2.06, 2.05 and 2.01 (3s, 9H, 3xC=OCH$_3$); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 170.5 and 169.8 (3xC=O), 84.2 (C-1), 72.2 (C-3), 68.8 (C-4), 67.9 (C-5), 62.0 (C-6), 61.7 (C-2), 20.73, 20.70 and 20.6 (3xC=OCH$_3$), 12.9 (SCH$_3$); HRMS (ES+) Calcd for $C_{13}H_{19}N_3O_7SNa$ 384.0841, found 384.0840 [M+Na]$^+$; Anal. Calcd for $C_{13}H_{19}N_3O_7S$: C, 43.21; H, 5.30; N, 11.63. Found: C, 43.50; H, 5.25; N, 11.50.

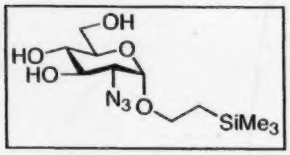
***(2-Trimethylsilyl)ethyl 3,4,6-tri-O-acetyl-2-azido-2-deoxy-α-D-glucopyranoside* (56).**



The known compound **56**^{390} was prepared using the method described by Pozsgay.^{390} To a solution of **55** (8.60 g, 23.81 mmol) in anhydrous CH$_2$Cl$_2$ (173 mL) at rt was added bromine (2.45 mL, 47.62 mmol). After 5 min, 1-hexene (9 mL) was added to the solution, and then, most of the volatiles were removed *in vacuo*. The residual syrup was treated with 2-(trimethylsilyl)ethanol (51.8 mL) followed by *N,N*-diisopropylethylamine (8.3 mL, 47.62 mmol). After 30 h the solution was concentrated almost to dryness and the residue was purified by flash chromatography (SiO$_2$; 5:3/hexane:EtOAc, R_f 0.46) to give **56** (9.33 g, 91%) as a pale yellow oil: $[\alpha]_D^{26}$ +395° (*c* 0.34, CHCl$_3$) (lit.^{390} $[\alpha]_D^{26}$ +127° (*c* 0.91, CHCl$_3$)); IR (CHCl$_3$ cast) 2954, 2109, 1752, 1367, 1227, 1148, 1048, 861, 839 cm^{-1}; ^{1}H NMR (CDCl$_3$, 300 MHz) (lit.^{390}) δ 5.46 (dd, 1H, $J_{3,2}$ = 10.5 Hz, $J_{3,4}$ = 9.3 Hz, CH-3), 4.99 (dd, 1H, $J_{4,5}$ = 10.2 Hz, $J_{4,3}$ = 9.3 Hz, CH-4), 4.96 (d, 1H, $J_{1,2}$ = 3.6 Hz, CH-1), 4.22 (dd, 1H, $J_{6,6'}$ = 12.3 Hz, $J_{6,5}$ = 5.1 Hz, CH-6), 4.06 (dd, 1H, $J_{6',6}$ = 12.3 Hz, $J_{6',5}$ = 2.1 Hz, CH-6'), 4.01 (ddd, 1H, $J_{5,4}$ = 10.2 Hz, $J_{5,6}$ = 5.1 Hz, $J_{5,6'}$ = 2.1 Hz, CH-5), 3.78 (ddd, 1H, $J_{1',1''}$ =

11.4 Hz, $J_{1',2'}$ = 9.9 Hz, $J_{1',2''}$ = 6.0 Hz, C$\underline{H}$-1'), 3.57 (ddd, 1H, $J_{1'',1'}$ = 11.4 Hz, $J_{1'',2''}$ = 9.9 Hz, $J_{1'',2'}$ = 6.0 Hz, C$\underline{H}$-1"), 3.27 (dd, 1H, $J_{2,3}$ = 10.5 Hz, $J_{2,1}$ = 3.6 Hz, C$\underline{H}$-2), 2.05, 2.04 and 2.01 (3s, 9H, 3xC=OC$\underline{H}_3$), 1.05-0.98 (m, 2H, C$\underline{H}_2$Si(CH$_3$)$_3$), 0.01 (s, 9H, Si(C$\underline{H}_3$)$_3$); ^{13}C NMR (CDCl$_3$, 75 MHz) (lit.[390]) δ 170.6, 170.0 and 169.7 (3x$\underline{C}$=O), 97.2 ($\underline{C}$-1), 70.6 ($\underline{C}$-3), 68.8 ($\underline{C}$-4), 67.6 ($\underline{C}$-5), 66.5 ($\underline{C}$-1'), 62.1 ($\underline{C}$-6), 60.8 ($\underline{C}$-2), 20.7 and 20.6 (3xC=O$\underline{C}$H$_3$), 18.1 ($\underline{C}$-2'), -1.5 (Si($\underline{C}$H$_3$)$_3$); HRMS (ES+) Calcd for $C_{17}H_{29}N_3O_8SiNa$ 454.1622, found 454.1623 [M+Na]$^+$; Anal. Calcd for $C_{17}H_{29}N_3O_8Si$: C, 47.32; H, 6.77; N, 9.74. Found: C, 47.06; H, 6.81; N, 9.59.

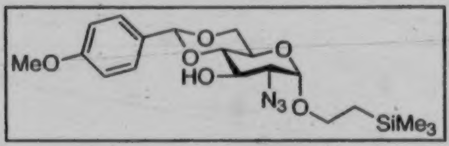
***(2-Trimethylsilyl)ethyl 2-azido-2-deoxy-α-D-glucopyranoside* (57).**



This deacetylation was done using a method described by Wessel.[391] A solution of **56** (9.13 g, 21.17 mmol) in dry MeOH (200 mL) was stirred in the presence of sodium methanolate (2.12 mL of a 1 M solution, 10% eq., 2.12 mmol) at rt for 12 h. The reaction mixture was then neutralized with Amberlite IR-120 (H$^+$) resin, filtered, and concentrated under reduced pressure. Further purification by recrystallization in Et$_2$O:hexane gave **57** (5.49 g, 85%) as a white solid: mp 127-128 °C; $[\alpha]_D^{26}$ +127° (*c* 0.56, CHCl$_3$); IR (CHCl$_3$ cast) 3414, 2953, 2925, 2107, 1314, 1248, 1142, 1085, 1056, 1020, 858, 835, 762 cm^{-1}; ^{1}H NMR (CD$_3$OD, 300 MHz) δ 4.87 (d, 1H, $J_{1,2}$ = 3.6 Hz, C$\underline{H}$-1), 3.87 (dt, 1H, $J_{1',1''}$ = 9.6 Hz, $J_{1',2'}$ = 6.6 Hz, C$\underline{H}$-1'), 5.46 (dd, 1H, $J_{3,2}$ = 10.2 Hz, $J_{3,4}$ = 8.7 Hz, C$\underline{H}$-3), 3.79 (dd, 1H, $J_{6,6'}$ = 11.7 Hz, $J_{6,5}$ = 2.4 Hz, C$\underline{H}$-6), 3.67 (dd, 1H, $J_{6',6}$ = 11.7 Hz, $J_{6',5}$ = 5.4 Hz, C$\underline{H}$-6'), 3.59 (ddd, 1H, $J_{5,4}$ = 9.6 Hz, $J_{5,6}$ = 5.4 Hz, $J_{5,6'}$ = 2.4 Hz, C$\underline{H}$-5), 3.57 (dt, 1H, $J_{1'',1'}$ = 9.3 Hz,

$J_{1'',2'}$ = 6.6 Hz, C$\underline{H}$-1"), 3.32 (dd, 1H, $J_{4,5}$ = 9.6 Hz, $J_{4,3}$ = 8.7 Hz, C$\underline{H}$-4), 3.07 (dd, 1H, $J_{2,3}$ = 10.2 Hz, $J_{2,1}$ = 3.6 Hz, C$\underline{H}$-2), 1.07-0.89 (m, 2H, C$\underline{H}_2$Si(CH_3)$_3$), 0.05 (s, 9H, Si(C$\underline{H}_3$)$_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 97.5 ($\underline{C}$-1), 71.8 ($\underline{C}$-5), 71.3 ($\underline{C}$-3), 70.4 ($\underline{C}$-4), 66.1 ($\underline{C}$-6), 62.8 ($\underline{C}$-2), 61.4 ($\underline{C}$-1'), 18.1 ($\underline{C}$-2'), -1.4 (Si($\underline{C}H_3$)$_3$); HRMS (ES+) Calcd for $C_{11}H_{23}N_3O_5SiNa$ 328.1305, found 328.1311 [M+Na]$^+$; Anal. Calcd for $C_{11}H_{23}N_3O_5Si$: C, 43.26; H, 7.59; N, 13.76. Found: C, 43.33; H, 7.64; N, 13.59.

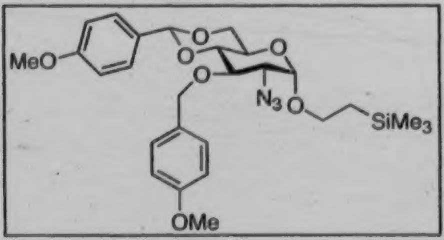
***(2-Trimethylsilyl)ethyl 2-azido-2-deoxy-4,6-*O*-(p-methoxybenzylidene)-α-*D*-glucopyranoside* (58).**



To a solution of (3.64 g, 11.92 mmol) and anisaldehyde dimethylacetal (7.96 mL, 46.75 mmol) in dry MeCN (77 mL) was added, at rt, a catalytic amount of *p*-TsOH (23 mg). After being stirred for 3 h the reaction mixture was quenched with Et_3N, concentrated *in vacuo*, and the residue obtained was purified by flash chromatography (SiO_2; 5:1/hexane:EtOAc, R_f 0.12) to give **58** (4.80 g, 95%) as a white solid: $[\alpha]_D^{26}$ +106° (*c* 0.59, $CHCl_3$); IR ($CHCl_3$ cast) 3457, 2953, 2109, 1615, 1519, 1466, 1377, 1251, 1057, 1032, 990, 860, 834 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.40 (dt, 2H, J = 9.3 Hz, J = 2.7 Hz, C$\underline{H}$-2 of PMB), 6.88 (dt, 2H, J = 9.3 Hz, J = 2.7 Hz, C$\underline{H}$-3 of PMB), 5.48 (s, 1H, OC$\underline{H}$O), 4.90 (d, 1H, $J_{1,2}$ = 3.6 Hz, C$\underline{H}$-1), 4.24 (dd, 1H, $J_{6,6'}$ = 10.2 Hz, $J_{6,5}$ = 4.8 Hz, C$\underline{H}$-6), 4.20 (dd, 1H, $J_{3,2}$ = 9.9 Hz, $J_{3,4}$ = 9.3 Hz, C$\underline{H}$-3), 3.86 (dt, $J_{5,4}$ = 9.9 Hz, $J_{5,6}$ = 4.8 Hz, C$\underline{H}$-5), 3.80 (ddd, 1H, $J_{1',1''}$ = 10.8 Hz, $J_{1',2'}$ = 9.9 Hz, $J_{1',2''}$ = 6.0 Hz, C$\underline{H}$-1'), 3.79 (s, 3H, OC$\underline{H}_3$), 3.70 (t, 1H, $J_{6',6}$ = 10.2 Hz, C$\underline{H}$-6'), 3.55 (ddd, 1H, $J_{1'',1'}$ =

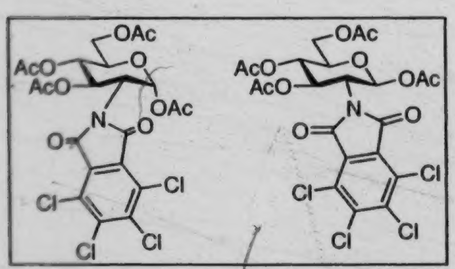
10.8 Hz, $J_{1'',2''}$ = 9.9 Hz, $J_{1'',1'}$ = 6.0 Hz, CH-1"), 3.47 (t, 1H, $J_{4,3}$ = 9.3 Hz, CH-4), 3.25 (dd, 1H, $J_{2,3}$ = 9.9 Hz, $J_{2,1}$ = 3.6 Hz, CH-2), 2.65 (br s, 1H, OH), 1.11-0.93 (m, 2H, $CH_2Si(CH_3)_3$), 0.03 (s, 9H, $Si(CH_3)_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 161.3 (C-1 of PMB), 130.5 (C-4 of PMB), 128.6 (C-2 of PMB), 114.8 (C-3 of PMB), 103.0 (OCHO), 99.0 (C-1), 82.9 (C-4), 70.0 (C-3), 69.9 (C-6), 67.3 (C-1'), 64.1 (C-2), 63.4 (C-5), 56.3 (OCH_3), 19.1 (C-2'), -0.4 ($Si(CH_3)_3$); HRMS (ES+) Calcd for $C_{19}H_{29}N_3O_6SiNa$ 446.1723, found 446.1722 $[M+Na]^+$.

***(2-Trimethylsilyl)ethyl 2-azido-2-deoxy-3-O-(p-methoxybenzyl)-4,6-O-(p-methoxybenzylidene)-α-D-glucopyranoside* (59).**



This *p*-methoxybenzylation was accomplished using a method described by Jenkins.[392] A solution of **58** (4.74 g, 11.19 mmol) in dry DMF (24 mL) was treated with NaH (538 mg of a 60% w/w dispersion in oil, 13.44 mmol). The solution was stirred at rt for 30 min, and *p*-methoxybenzyl chloride (1.60 mL, 11.75 mmol) was added dropwise. The mixture was stirred for 23 h. Methanol (5 mL) was added, and stirring was continued for 1 h. The solvents were evaporated under reduced pressure, and the residue was extracted with $CHCl_3$ (3x75 mL). The combined organic extracts were washed with H_2O (2x50 mL), dried ($MgSO_4$), filtered, and concentrated *in vacuo*. The residue obtained was purified by flash chromatography (SiO_2; 5:1/hexane:EtOAc, R_f 0.18) to give **59** (5.75 g, 95%) as a colorless oil: $[\alpha]_D^{26}$ +49° (*c* 0.27, $CHCl_3$); IR ($CHCl_3$ cast) 3485, 2953, 2837, 2107, 1614, 1515, 1465, 1250, 1172, 1092, 1036, 834 cm^{-1}; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 7.37 (dt, 2H, J = 9.6 Hz, J = 2.7 Hz, $C\underline{H}$-2 of PMB), 7.28 (dt, 2H, J = 9.6 Hz, J = 2.1 Hz, $C\underline{H}$-2 of PMB), 6.90 (dt, 2H, J = 9.6 Hz, J = 2.7 Hz, $C\underline{H}$-3 of PMB), 6.83 (dt, 2H, J = 9.6 Hz, J = 2.1 Hz, $C\underline{H}$-3 of PMB), 5.52 (s, 1H, $OC\underline{H}O$), 4.87 (d, 1H, $J_{1,2}$ = 3.6 Hz, $C\underline{H}$-1), 4.84 (d, 1H, $J_{4',4''}$ = 10.5 Hz, $C\underline{H}$-4' part of $OCH_2C_6H_5$), 4.71 (d, 1H, $J_{4'',4'}$ = 10.5 Hz, $C\underline{H}$-4" part of $OCH_2C_6H_5$), 4.24 (dd, 1H, $J_{6,6'}$ = 10.2 Hz, $J_{6,5}$ = 4.8 Hz, $C\underline{H}$-6), 4.03 (dd, 1H, $J_{3,2}$ = 9.9 Hz, $J_{3,4}$ = 9.3 Hz, $C\underline{H}$-3), 3.86 (ddd, 1H, $J_{5,4}$ = 10.2 Hz, $J_{5,6}$ = 4.8 Hz, $J_{5,6'}$ = 2.4 Hz, $C\underline{H}$-5), 3.81 and 3.77 (2s, 6H, 2x$OC\underline{H}_3$), 3.77 (ddd, 1H, $J_{1',1''}$ = 11.1 Hz, $J_{1',2'}$ = 9.9 Hz, $J_{1',2''}$ = 5.7 Hz, $C\underline{H}$-1'), 3.70 (d, 1H, $J_{6',6}$ = 10.2 Hz, $C\underline{H}$-6'), 3.64 (t, 1H, $J_{4,3}$ = 9.3 Hz, $C\underline{H}$-4), 3.53 (ddd, 1H, $J_{1'',1'}$ = 11.1 Hz, $J_{1'',2''}$ = 9.9 Hz, $J_{1'',2'}$ = 5.7 Hz, $C\underline{H}$-1"), 3.33 (dd, 1H, $J_{2,3}$ = 9.9 Hz, $J_{2,1}$ = 3.6 Hz, $C\underline{H}$-2), 1.10-0.91 (m, 2H, $C\underline{H}_2Si(CH_3)_3$), 0.02 (s, 9H, $Si(C\underline{H}_3)_3$); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 160.1 and 159.4 (2x$\underline{C}$-1 of PMB), 130.1 and 129.9 (2x$\underline{C}$-4 of PMB), 130.0 and 127.4 (2x$\underline{C}$-2 of PMB), 113.9 and 113.7 (2x$\underline{C}$-3 of PMB), 101.5 ($O\underline{C}HO$), 98.0 ($\underline{C}$-1), 89.9 ($\underline{C}$-4), 76.0 ($\underline{C}$-3), 74.7 ($O\underline{C}H_2C_6H_5$), 69.0 ($\underline{C}$-6), 66.1 ($\underline{C}$-1'), 63.1 ($\underline{C}$-2), 62.8 ($\underline{C}$-5), 55.4 and 55.3 (2x$O\underline{C}H_3$), 18.2 ($\underline{C}H_2Si(CH_3)_3$), -1.4 ($Si(\underline{C}H_3)_3$); HRMS (ES+) Calcd for $C_{27}H_{37}N_3O_7SiNa$ 566.2298, found 566.2302 [M+Na]$^+$.

***1,3,4,6-Tetra-O-acetyl-2-deoxy-2-tetrachlorophthalimido-α-D-glucopyranoside* (60) *and 1,3,4,6-tetra-O-acetyl-2-deoxy-2-tetrachlorophthalimido-β-D-glucopyranoside* (61).**

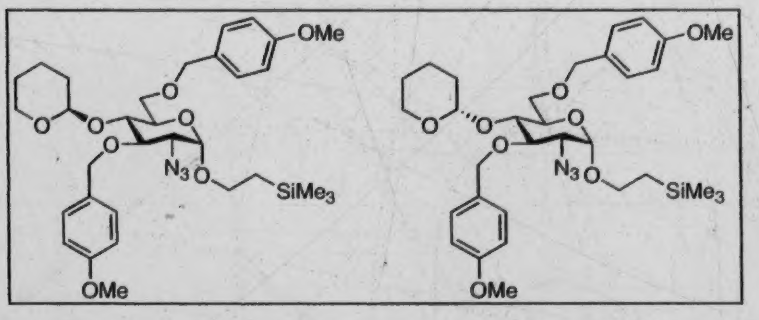


The known compounds **60**[183,202] and **61**[183,393,394] were isolated and characterized individually, but prepared and used as a α:β mixture according to the method described by Castro-Palomino.[183] *D*-glucosamine hydrochloride (**53**) (5 g, 23.15 mmol) was added to a sodium methoxide solution prepared from sodium (0.532 g, 23.15 mmol) and MeOH (24 mL). After 10 min the mixture was treated with 3,4,5,6-tetrachlorophthalic anhydride (3.310 g, 11.57 mmol) and stirred for 20 min. Et_3N (2.4 mL, 23.15 mmol) was added and the reaction mixture was again treated with 3,4,5,6-tetrachlorophthalic anhydride (3.310 g, 11.57 mmol). The mixture was then warmed to 50 °C and stirred for 20 min. After concentration of the mixture, the residue was treated with pyridine (46 mL) and acetic anhydride (46 mL) at rt overnight. The reaction mixture was poured into iced-cold H_2O and extracted with $CHCl_3$ (3x70 mL). The organic combined organic layers were successively washed with H_2O, 3% aq. HCl, a saturated aq. $NaHCO_3$ solution, and H_2O, dried (Na_2SO_4), and concentrated under reduced pressure. Further purification by flash chromatography (SiO_2, 2:1/hexane:EtOAc, R_f**60** 0.24 and R_f**61** 0.25) gave **60** and **61** (5.62 g, 79%, α:β/4:1) as white solids.

Data for **60**: mp 228-229 °C (lit.[202] mp 219.5 °C); $[\alpha]_D^{26}$ +94° (*c* 0.63, $CHCl_3$) (lit.[202] $[\alpha]_D^{20}$ +119° (*c* 1.00, $CHCl_3$)); IR ($CHCl_3$ cast) 2955, 1752, 1728, 1652, 1386, 1217, 1049, 911, 753, 739 cm^{-1}; ^{1}H NMR (CDCl$_3$, 300 MHz) (lit.[183,202]) δ 6.47 (dd, 1H, $J_{3,2}$ = 11.7 Hz, $J_{3,4}$ = 9.0 Hz, C$\underline{\text{H}}$-3), 6.24 (d, 1H, $J_{1,2}$ = 3.6 Hz, C$\underline{\text{H}}$-1), 5.15 (dd, 1H, $J_{4,5}$ = 10.2 Hz, $J_{4,3}$ = 9.0 Hz, C$\underline{\text{H}}$-4), 4.69 (dd, 1H, $J_{2,3}$ = 11.7 Hz, $J_{2,1}$ = 3.6 Hz, C$\underline{\text{H}}$-2), 4.35 (dd, 1H, $J_{6,6'}$ = 12.3 Hz, $J_{6,5}$ = 3.9 Hz, C$\underline{\text{H}}$-6), 4.31-4.28 (m, 1H, C$\underline{\text{H}}$-5), 4.13 (dd, 1H, $J_{6',6}$ = 12.3 Hz, $J_{6',5}$ = 2.1 Hz, C$\underline{\text{H}}$-6'), 2.11, 2.08, 2.04 and 1.89 (4s, 12H, 4xC=OC$\underline{\text{H}}_3$); ^{13}C NMR (CDCl$_3$, 75 MHz) (lit.[202]) δ 170.6, 169.7, 169.6, 169.5 and 162.7 (6x$\underline{\text{C}}$=O), 140.8, 130.2 and 126.7 (3x$\underline{\text{C}}$=$\underline{\text{C}}$), 90.5 ($\underline{\text{C}}$-1), 70.3 ($\underline{\text{C}}$-5), 69.3 ($\underline{\text{C}}$-4), 67.0 ($\underline{\text{C}}$-3), 61.5 ($\underline{\text{C}}$-6), 53.5 ($\underline{\text{C}}$-2), 21.0, 20.75, 20.71 and 20.6 (4xC=O$\underline{\text{C}}$H$_3$); HRMS (ES+) Calcd for $C_{22}H_{19}Cl_4NO_{11}Na$ 635.9610, found 635.9608 [M+Na]$^+$; Anal. Calcd for $C_{22}H_{19}Cl_4NO_{11}$: C, 42.95; H, 3.11; N, 2.28. Found: C, 42.99; H, 2.96; N, 2.24.

Data for **61**: (lit.[394] mp 192-196 °C) (lit.[393] mp 169-171 °C); (lit.[393] $[\alpha]_{\text{D}}^{20}$ +69.7° (*c* 0.9, CHCl$_3$)); IR (CHCl$_3$ cast) 2955, 1752, 1728, 1652, 1386, 1217, 1049, 911, 753, 739 cm^{-1}; ^{1}H NMR (CDCl$_3$, 300 MHz) (lit.[183,393,394]) δ 6.41 (d, 1H, $J_{1,2}$ = 8.7 Hz, C$\underline{\text{H}}$-1), 5.74 (dd, 1H, $J_{3,2}$ = 10.5 Hz, $J_{3,4}$ = 9.3 Hz, C$\underline{\text{H}}$-3), 5.17 (dd, 1H, $J_{4,5}$ = 10.5 Hz, $J_{4,3}$ = 9.3 Hz, C$\underline{\text{H}}$-4), 4.39 (dd, 1H, $J_{2,3}$ = 10.5 Hz, $J_{2,1}$ = 8.7 Hz, C$\underline{\text{H}}$-2), 4.30 (d, 1H, $J_{6,6'}$ = 10.2 Hz, C$\underline{\text{H}}$-6), 4.27 (d, 1H, $J_{6',6}$ = 10.2 Hz, C$\underline{\text{H}}$-6'), 3.94 (ddd, $J_{5,4}$ = 10.2 Hz, $J_{5,6}$ = 4.5 Hz, $J_{5,6'}$ = 2.1 Hz, C$\underline{\text{H}}$-5), 2.05, 1.99, 1.98 and 1.84 (4s, 12H, 4xC=OC$\underline{\text{H}}_3$); ^{13}C NMR (CDCl$_3$, 75 MHz) (lit.[393,394]) δ 170.4, 169.6, 169.35, 169.27 and 168.5 (6x$\underline{\text{C}}$=O), 140.7, 130.1 and 126.8 (3x$\underline{\text{C}}$=$\underline{\text{C}}$), 89.6 ($\underline{\text{C}}$-1), 72.6 ($\underline{\text{C}}$-5), 70.7 ($\underline{\text{C}}$-4), 67.9 ($\underline{\text{C}}$-3), 61.4 ($\underline{\text{C}}$-6), 54.3 ($\underline{\text{C}}$-2), 20.62, 20.56, 20.52 and 20.4 (4xC=O$\underline{\text{C}}$H$_3$); HRMS (ES+) Calcd for $C_{22}H_{19}Cl_4NO_{11}Na$ 635.9610, found 635.9608 [M+Na]$^+$.

***(2-Trimethylsilyl)ethyl 2-azido-2-deoxy-3,6-di-*O*-(*p*-methoxybenzyl)-4-(*R*)-tetrahydropyran-α-*D*-glucopyranoside* (65) *and (2-trimethylsilyl)ethyl 2-azido-2-deoxy-3,6-di-*O*-(*p*-methoxybenzyl)-4-(*S*)-tetrahydropyran-α-*D*-glucopyranoside* (66).**



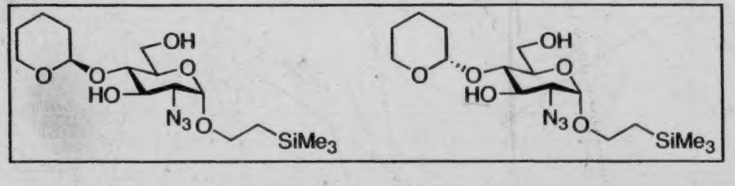
This protection was performed using a method described by Miyashita.[382] A solution of alcohol **50** (3.161 g, 5.80 mmol) and dihydropyran (1.1 mL, 11.6 mmol) in dry CH_2Cl_2 (41 mL) containing PPTS (145 mg, 0.58 mmol) was stirred at rt for 9.5 h. The reaction mixture was then diluted with CH_2Cl_2, washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure. Further purification by flash chromatography (SiO_2, 3:1/hexane:EtOAc, R_f**65** 0.28 and R_f**66** 0.33) gave **65** and **66** (3.56 g, 98%) as colorless oils.

Data for **65**: $[\alpha]_D^{26}$ +86° (*c* 0.11, $CHCl_3$); IR ($CHCl_3$ cast) 2949, 2105, 1613, 1514, 1465, 1441, 1248, 1173, 1033, 836 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.28 (dt, 2H, *J* = 8.7 Hz, *J* = 2.1 Hz, C$\underline{H}$-2 of PMB), 7.23 (dt, 2H, *J* = 8.7 Hz, *J* = 2.1 Hz, C$\underline{H}$-2 of PMB), 6.86 (dt, 2H, *J* = 8.7 Hz, *J* = 2.1 Hz, C$\underline{H}$-3 of PMB), 6.82 (dt, 2H, *J* = 8.7 Hz, *J* = 2.1 Hz, C$\underline{H}$-3 of PMB), 4.91 (d, 1H, $J_{1,2}$ = 3.6 Hz, C$\underline{H}$-1), 4.79 (d, 1H, $J_{4',4''}$ = 10.2 Hz, C$\underline{H}$-4' part of $OCH_2C_6H_5$), 4.78-4.74 (m, 1H, OC$\underline{H}$ of THP), 4.63 (d, 1H, $J_{4'',4'}$ = 10.2 Hz, C$\underline{H}$-4" part of $OCH_2C_6H_5$), 4.51 (d, 1H, $J_{4',4''}$ = 11.4 Hz, C$\underline{H}$-4' part of $OCH_2C_6H_5$), 4.43 (d, 1H, $J_{4'',4'}$ = 11.4 Hz, C$\underline{H}$-4" part of $OCH_2C_6H_5$), 3.90 (dd, 1H, $J_{3,2}$ = 10.2 Hz, $J_{3,4}$ = 8.4 Hz, C$\underline{H}$-3), 3.82-3.64 (m, 5H, 1H of OC$\underline{H}_2$ of THP, C$\underline{H}_2$-6 and OC$\underline{H}_2$$CH_2Si(CH_3)_3$), 3.78 and 3.77

(2s, 6H, 2xOC$\underline{H}_3$), 3.69 (dd, 1H, $J_{4,5}$ = 11.1 Hz, $J_{4,3}$ = 8.4 Hz, C$\underline{H}$-4), 3.52 (ddd, 1H, $J_{5,4}$ = 11.1 Hz, $J_{5,6}$ = 9.9 Hz, $J_{5,6'}$ = 6.0 Hz, C$\underline{H}$-5), 3.37-3.28 (m, 1H, 1H of OC$\underline{H}_2$ of THP), 3.30 (dd, 1H, $J_{2,3}$ = 10.2 Hz, $J_{2,1}$ = 3.6 Hz, C$\underline{H}$-2), 1.80-1.66 (m, 2H, OCHCH$_2$C$\underline{H}_2$ of THP), 1.45-1.39 (m, 4H, OCHC$\underline{H}_2$CH$_2$C$\underline{H}_2$ of THP), 1.10-0.90 (m, 2H, C$\underline{H}_2$Si(CH$_3$)$_3$), -0.01 (s, 9H, Si(C$\underline{H}_3$)$_3$); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 159.5 and 159.1 (2x$\underline{C}$-1 of PMB), 130.8 and 130.2 (2x$\underline{C}$-4 of PMB), 129.9, 129.7, 129.4 and 129.2 (2x$\underline{C}$-2 and $\underline{C}$-6 of PMB), 114.1, 114.0, 113.9 and 113.7 (2x$\underline{C}$-3 and $\underline{C}$-5 of PMB), 101.7 (O$\underline{C}$H of THP), 96.9 ($\underline{C}$-1), 80.6 ($\underline{C}$-5), 75.8 and 70.7 ($\underline{C}$-3 and $\underline{C}$-4), 75.0 and 73.4 (2x$\underline{C}$H$_2$C$_6$H$_5$), 68.9, 65.5 and 64.7 (O$\underline{C}$H$_2$ of THP, $\underline{C}$-6 and O$\underline{C}$H$_2$CH$_2$Si(CH$_3$)$_3$), 63.6 ($\underline{C}$-2), 55.3 (2xO$\underline{C}$H$_3$), 31.7, 25.3 and 21.2 (OCH($\underline{C}$H$_2$)$_3$ of THP), 18.1 ($\underline{C}$H$_2$Si(CH$_3$)$_3$), -1.4 (Si($\underline{C}$H$_3$)$_3$); HRMS (ES+) Calcd for $C_{32}H_{47}N_3O_8SiNa$ 652.3025, found 652.3026 [M+Na]$^+$.

Data for **66**: $[\alpha]_D^{26}$ +48° (c 0.66, CHCl$_3$); IR (CHCl$_3$ cast) 2949, 2106, 1613, 1514, 1465, 1441, 1248, 1173, 1034, 837 cm^{-1}; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 7.36 (dt, 2H, J = 8.7 Hz, J = 2.4 Hz, C$\underline{H}$-2 of PMB), 7.23 (dt, 2H, J = 9.0 Hz, J = 2.1 Hz, C$\underline{H}$-2 of PMB), 6.85-6.80 (m, 4H, 2xC$\underline{H}$-3 of PMB), 4.94 (d, 1H, $J_{4',4''}$ = 10.5 Hz, C$\underline{H}$-4' part of OCH$_2$C$_6$H$_5$), 4.86 (d, 1H, $J_{1,2}$ = 3.6 Hz, C$\underline{H}$-1), 4.64 (d, 1H, $J_{4'',4'}$ = 9.6 Hz, C$\underline{H}$-4" part of OCH$_2$C$_6$H$_5$), 4.56 (d, 1H, $J_{4',4''}$ = 12.0 Hz, C$\underline{H}$-4' part of OCH$_2$C$_6$H$_5$), 4.48-4.41 (m, 1H, OC$\underline{H}$ of THP), 4.37 (d, 1H, $J_{4'',4'}$ = 12.0 Hz, C$\underline{H}$-4" part of OCH$_2$C$_6$H$_5$), 3.96-3.46 (m, 9H, C$\underline{H}$-3, C$\underline{H}$-4, C$\underline{H}$-5, 1H of OC$\underline{H}_2$ of THP, C$\underline{H}_2$-6 and OC$\underline{H}_2$CH$_2$Si(CH$_3$)$_3$), 3.35 (dd, 1H, $J_{2,3}$ = 9.6 Hz, $J_{2,1}$ = 3.6 Hz, C$\underline{H}$-2), 3.34-3.23 (m, 1H, 1H of OC$\underline{H}_2$ of THP), 1.80-1.68 (m, 2H, OCHCH$_2$C$\underline{H}_2$ of THP), 1.48-1.32 (m, 4H, OCHC$\underline{H}_2$CH$_2$C$\underline{H}_2$ of THP), 1.09-0.87 (m, 2H, C$\underline{H}_2$Si(CH$_3$)$_3$), -0.01 (s, 9H, Si(C$\underline{H}_3$)$_3$); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 159.3 and 159.2 (2x$\underline{C}$-1 of PMB), 130.6 and 130.0 (2x$\underline{C}$-4 of PMB), 130.2, 129.9, 129.6 and 129.4 (2x$\underline{C}$-2 and C-6 of PMB), 114.1, 113.9. 113.8 and 113.7 (2xC-3 and C-5 of PMB), 100.9 (OCH of THP), 97.1 (C-1), 78.7 (C-5), 76.0 and 70.9 (C-3 and C-4), 74.6 and 73.2 (2x$CH_2C_6H_5$), 68.1, 65.6 and 64.7 (OCH_2 of THP, C-6 and $OCH_2CH_2Si(CH_3)_3$), 63.2 (C-2), 55.3 (2xOCH_3), 31.5, 25.3 and 21.1 ($OCH(CH_2)_3$ of THP), 18.1 ($CH_2Si(CH_3)_3$), -1.4 ($Si(CH_3)_3$); HRMS (ES+) Calcd for $C_{32}H_{47}N_3O_8SiNa$ 652.3025, found 652.3024 $[M+Na]^+$.

***(2-Trimethylsilyl)ethyl 2-azido-2-deoxy-4-(*R*)-tetrahydropyran-α-D-glucopyranoside* (67) *and (2-trimethylsilyl)ethyl 2-azido-2-deoxy-4-(*S*)-tetrahydropyran-α-D-glucopyranoside* (68).**

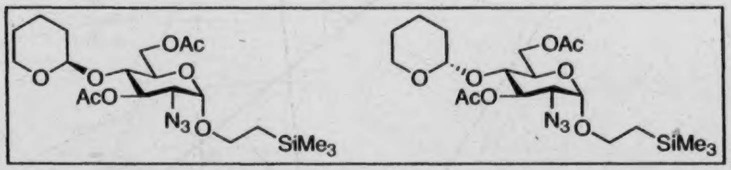


This deprotection was performed using the method described by Kloosterman.[189] To a solution of mixture of **65** and **66** (3.49 g, 5.54 mmol) and DDQ (3.94 g, 17.18 mmol) in dry CH_2Cl_2 (200 mL), H_2O (30.4 mL) was added in the dark under argon. After 3 h the organic layer was separated and the aqueous layer was extracted with CH_2Cl_2. The combined organic layer were washed with 5% aq. $NaHCO_3$, H_2O, dried (Na_2SO_4) and concentrated *in vacuo*. Further purification by flash chromatography (SiO_2, 8:92/MeOH: CH_2Cl_2, R_f**67** 0.55 and R_f**68** 0.62) gave **67** and **68** (2.26 g, quant.) as colorless oils.

Data for **67**: $[\alpha]_D^{26}$ +187° (*c* 0.18, $CHCl_3$); IR ($CHCl_3$ cast) 3403, 2950, 2107, 1350, 1250, 1138, 1029, 861, 837 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 4.90 (d, 1H, $J_{1,2}$ = 3.6 Hz, CH-1), 4.76-4.72 (m, 1H, OCH of THP), 4.11-4.04 (m, 1H, CH-3), 4.04-3.91 (m, 2H, CH-6 and 1H of $OC\underline{H}_2$ of THP), 3.76-3.60 (m, 3H, $C\underline{H}$-4, $C\underline{H}$-6' and $CHO\underline{H}$), 3.76 (ddd, 1H, $J_{1',1''}$ = 10.8 Hz, $J_{1',2'}$ = 9.9 Hz, $J_{1',2''}$ = 5.7 Hz, $C\underline{H}$-1'), 3.55-3.46 (m, 1H, 1H of $OC\underline{H}_2$ of THP), 3.52 (ddd, 1H, $J_{1'',1'}$ = 10.8 Hz, $J_{1'',2''}$ = 9.9 Hz, $J_{1'',2'}$ = 6.0 Hz, $C\underline{H}$-1"), 3.24-3.17 (m, 1H, $C\underline{H}$-5), 3.18 (dd, 1H, $J_{2,3}$ = 10.2 Hz, $J_{2,1}$ = 3.6 Hz, $C\underline{H}$-2), 2.60 (t, 1H, J = 3.6 Hz, $CH_2O\underline{H}$), 1.86-1.78 (m, 2H, $OCHCH_2C\underline{H}_2$ of THP), 1.55-1.44 (m, 4H, $OCHC\underline{H}_2CH_2C\underline{H}_2$ of THP), 1.06-0.86 (m, 2H, $C\underline{H}_2Si(CH_3)_3$), 0.00 (s, 9H, $Si(C\underline{H}_3)_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 102.7 ($O\underline{C}H$ of THP), 97.3 ($\underline{C}$-1), 75.9 ($\underline{C}$-4), 72.0 ($\underline{C}$-3), 70.7 ($\underline{C}$-5), 66.2 and 65.9 ($O\underline{C}H_2$ of THP and $\underline{C}$-6), 61.6 (C-1'), 63.5 ($\underline{C}$-2), 31.2, 25.0 and 21.7 ($OCH(\underline{C}H_2)_3$ of THP), 18.1 ($\underline{C}H_2Si(CH_3)_3$), -1.4 ($Si(\underline{C}H_3)_3$); HRMS (ES+) Calcd for $C_{16}H_{31}N_3O_6SiNa$ 412.1874, found 412.1872 $[M+Na]^+$.

Data for **68**: $[\alpha]_D^{26}$ +108° (c 0.37, $CHCl_3$); IR ($CHCl_3$ cast) 3409, 2950, 2109, 1613, 1352, 1317, 1250, 1162, 1139, 1026, 860, 837 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.01 (s, 1H, $O\underline{H}$), 4.83 (d, 1H, $J_{1,2}$ = 3.6 Hz, $C\underline{H}$-1), 4.51-4.47 (m, 1H, $OC\underline{H}$ of THP), 4.08-4.00 (m, 1H, 1H of $OC\underline{H}_2$ of THP), 4.00 (dd, 1H, $J_{3,2}$ = 10.2 Hz, $J_{3,4}$ = 8.4 Hz, $C\underline{H}$-3), 3.81-3.65 (m, 4H, $C\underline{H}$-5, $C\underline{H}_2$-6 and 1H of $OC\underline{H}_2CH_2Si(CH_3)_3$), 3.60-3.46 (m, 3H, $C\underline{H}$-4, 1 H of $OC\underline{H}_2$ of THP and 1H of $OC\underline{H}_2CH_2Si(CH_3)_3$), 3.13 (dd, 1H, $J_{2,3}$ = 10.2 Hz, $J_{2,1}$ = 3.6 Hz, $C\underline{H}$-2), 1.86-1.50 (3m, 6H, $OCH_2(C\underline{H}_2)_3CH$), 1.07-0.88 (m, 2H, $C\underline{H}_2Si(CH_3)_3$), 0.01 (s, 9H, $Si(C\underline{H}_3)_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 102.2 ($O\underline{C}H$ of THP), 97.4 ($\underline{C}$-1), 80.8 ($\underline{C}$-5), 70.1 ($\underline{C}$-3 and $\underline{C}$-4), 66.1, 65.6 and 61.6 ($O\underline{C}H_2$ of THP, $\underline{C}$-6 and $O\underline{C}H_2CH_2Si(CH_3)_3$), 63.0 ($\underline{C}$-2), 31.4, 24.9 and 21.1 ($OCH(\underline{C}H_2)_3$ of THP), 18.1 ($\underline{C}H_2Si(CH_3)_3$), -1.4 ($Si(\underline{C}H_3)_3$); HRMS (ES+) Calcd for $C_{16}H_{31}N_3O_6SiNa$ 412.1874, found 412.1872 $[M+Na]^+$.

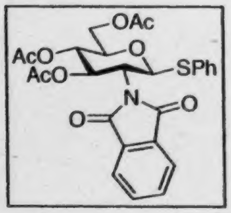
***(2-Trimethylsilyl)ethyl 2-azido-2-deoxy-3,6-di-O-acetyl-4-(R)-tetrahydropyran-α-D-glucopyranoside* (69) *and (2-trimethylsilyl)ethyl 2-azido-2-deoxy-3,6-di-O-acetyl-4-(S)-tetrahydropyran-α-D-glucopyranoside* (70).**



Pyridine (60 mL) and Ac_2O (60 mL) were added to a mixture of **67** and **68** (1.986 g, 5.10 mmol). The mixture was stirred overnight at rt under argon, diluted with CH_2Cl_2, washed with a 6 N HCl solution, a saturated aq. $NaHCO_3$ solution, H_2O, and brine, dried (Na_2SO_4), and concentrated *in vacuo*. Further purification by flash chromatography (SiO_2; 2:1/hexane:EtOAc, R_f 0.34) gave a mixture of diastereoisomers **69** and **70** (1.70 g, 86%) as a colorless oil: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.49 and 5.46 (2dd, 2H, $J_{3,2}$ = 10.8 Hz, $J_{3,4}$ = 8.7 Hz, 2xC$\underline{H}$-3), 4.95 and 4.93 (2d, 2H, $J_{1,2}$ = 3.6 Hz, 2xC$\underline{H}$-1), 4.58-4.54 and 4.50-4.45 (2m, 2H, 2xOC$\underline{H}$ of THP), 4.35-4.18 (m, 2H, 2x1H of OC$\underline{H}_2$ of THP), 3.98-3.90, 3.87-3.64, 3.61-3.51 and 3.47-3.36 (4m, 14H, 2x(C$\underline{H}$-4, C$\underline{H}$-5, C$\underline{H}_2$-6, 1H of OC$\underline{H}_2$ of THP and OC$\underline{H}_2$CH$_2$Si(CH$_3$)$_3$), 3.13 and 3.08 (2dd, 1H, $J_{2,3}$ = 10.8 Hz, $J_{2,1}$ = 3.6 Hz, 2xC$\underline{H}$-2), 2.10, 2.09, 2.07 and 2.05 (4s, 12H, 4xC=OC$\underline{H}_3$), 1.80-1.37 (m, 12H, 2xOCH(C$\underline{H}_2$)$_3$ of THP), 1.11-0.93 (m, 4H, 2xC$\underline{H}_2$Si(CH$_3$)$_3$), 0.011 and 0.009 (2s, 18H, 2xSi(C$\underline{H}_3$)$_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 170.8, 170.7, 170.2 and 169.7 (4x$\underline{C}$=O), 101.4 and 101.0 (2xO$\underline{C}$H of THP), 97.4 ($\underline{C}$-1), 76.4, 74.5, 72.8, 71.1, 69.0 and 68.8 (2x$\underline{C}$-3, 2x$\underline{C}$-4 and 2x$\underline{C}$-5), 66.2, 66.1, 64.2, 63.6, 63.4 and 62.9 (2xO$\underline{C}$H$_2$ of THP, 2x$\underline{C}$-6 and 2xO$\underline{C}$H$_2$CH$_2$Si(CH$_3$)$_3$), 61.2 and 61.7 (2x$\underline{C}$-2), 31.3, 31.2, 25.2, 25.1, 20.5 and

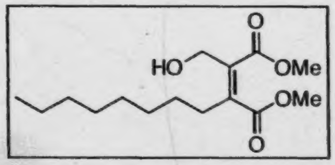
19.7 (2xOCH($\underline{C}H_2$)$_3$ of THP), 18.1 ($\underline{C}H_2Si(CH_3)_3$), -1.5 (Si($\underline{C}H_3$)$_3$); HRMS (ES+) Calcd for $C_{20}H_{35}N_3O_8SiNa$ 496.2091, found 496.2093 $[M+Na]^+$.

Phenyl 3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside (72).



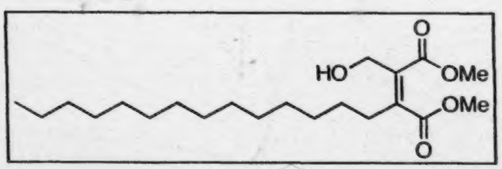
The known compound **72**[395-399] was prepared using the method described by Deng.[384] To a solution of **79** (297 mg, 0.62 mmol) in dry CH_2Cl_2 (2.5 mL) was added thiophenol (83 µL, 0.81 mmol) and $BF_3 \bullet Et_2O$ (0.77 mL, 6.07 mmol). After being stirred at rt under argon for 17 h, the reaction was quenched with a saturated aq. $NaHCO_3$ solution (7 mL), then extracted with CH_2Cl_2. The organic layers were washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure. The residue obtained was purified by flash chromatography (SiO_2; 2:1/hexane:EtOAc, R_f 0.11) to give **72** (245 mg, 75%) as a white solid: mp 145-146 °C (lit.[395,396] mp 145-146 °C); $[\alpha]_D^{26}$ +57° (*c* 0.23, $CHCl_3$) (lit.[396] $[\alpha]_D^{26}$ +56.1° (*c* 0.86, $CHCl_3$)) (lit.[400] $[\alpha]_D^{26}$ +60.9° (*c* 1.45, CH_2Cl_2)) (lit.[397] $[\alpha]_D^{26}$ +62.2° (*c* 1.8, $CHCl_3$)) (lit.[395] $[\alpha]_D^{26}$ +53° (*c* 1.4, $CHCl_3$)); IR ($CHCl_3$ cast) (lit.[397]) 2925, 1748, 1718, 1468, 1440, 1384, 1226, 1072, 1036, 720 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) (lit.[395-397,400]) δ 7.87-7.80, 7.76-7.69, 7.40-7.36 and 7.27-7.20 (4m, 9H, $C_6\underline{H}_5$ of SPh and $C_6\underline{H}_4$ of Phth), 5.77 (dd, 1H, $J_{3,2}$ = 10.2 Hz, $J_{3,4}$ = 9.3 Hz, C$\underline{H}$-3), 5.69 (d, 1H, $J_{1,2}$ = 10.8 Hz, C$\underline{H}$-1), 5.11 (dd, 1H, $J_{4,5}$ = 10.2 Hz, $J_{4,3}$ = 9.3 Hz, C$\underline{H}$-4), 4.33 (t, 1H, $J_{2,3}$ = 10.2 Hz, C$\underline{H}$-2), 4.26 (dd, 1H, $J_{6,6'}$ = 12.3 Hz, $J_{6,5}$ = 4.8 Hz, C$\underline{H}$-6), 4.18 (dd, 1H, $J_{6',6}$ = 12.3 Hz, $J_{6',5}$ = 2.4 Hz, C$\underline{H}$-6'), 3.87 (ddd, $J_{5,4}$ = 10.2 Hz, $J_{5,6}$ = 4.8 Hz, $J_{5,6'}$ = 2.4 Hz, C$\underline{H}$-5), 2.07, 1.99 and 1.80 (3s, 9H, 3xC=OC$\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 170.6, 170.1 and 169.4 (3x$\underline{C}$=OCH$_3$), 167.4 and 166.9 (2xN$\underline{C}$=O), 134.4, 133.3, 131.6, 131.2, 131.0, 128.9, 128.4, 123.7 (4x$\underline{C}$=$\underline{C}$), 83.1 ($\underline{C}$-1), 75.9 ($\underline{C}$-5), 71.6 ($\underline{C}$-3), 68.7 ($\underline{C}$-4), 62.2 ($\underline{C}$-6), 53.6 ($\underline{C}$-2), 20.7, 20.6 and 20.4 (3xC=O$\underline{C}H_3$); HRMS (ES+) Calcd for $C_{26}H_{25}NO_9SNa$ 550.1148, found 550.1153 $[M+Na]^+$; Anal. Calcd for $C_{26}H_{25}NO_9S$: C, 59.20; H, 4.78; N, 2.66. Found: C, 58.80; H, 4.36; N, 2.63.

Dimethyl (Z)-2-hydroxymethyl-3-octylbutenedioate **(73).**



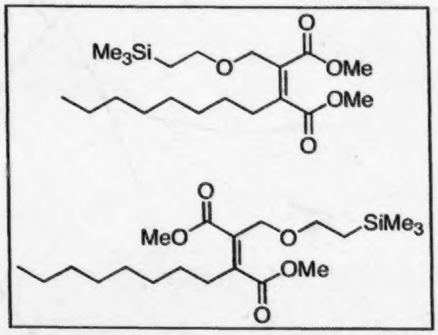
This deprotection reaction was performed using the method of Jansson.[191] To a solution of **75** (605 mg, 1.56 mmol) in dry MeCN (14 mL) was added $BF_3 \bullet Et_2O$ (0.17 mL, 1.41 mmol) at rt and the mixture was stirred for 2 h. The reaction was quenched by adding ice-cold H_2O (11 mL) and further stirred for 5 min. The aqueous layer was extracted with CH_2Cl_2, dried (Na_2SO_4), and concentrated *in vacuo*. Further purification by flash chromatography (SiO_2, 3:2/hexane:EtOAc, R_f 0.24) gave **73** (424 mg, 95%) as a colorless oil: IR ($CDCl_3$ cast) 3506, 2953, 2927, 2856, 1725, 1641, 1435, 1316, 1265, 1083 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 4.34 (s, 2H, $C\underline{H}_2OH$), 3.73 and 3.72 (2s, 6H, 2xOC$\underline{H}_3$), 2.43 (br s, 1H, $CH_2O\underline{H}$), 2.36 (t, 2H, J = 7.8 Hz, $CH_2C\underline{H}_2C$=C), 1.40 (qn, 2H, $C\underline{H}_2CH_2C$=C), 1.21 (br s, 10H, $(C\underline{H}_2)_5$), 0.83 (t, 3H, J = 6.8 Hz, $C\underline{H}_3$-chain); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 169.5 and 167.6 (2x$\underline{C}$=O), 143.9 and 132.7 ($\underline{C}$=$\underline{C}$), 58.8 ($\underline{C}H_2OH$), 52.3 and 52.2 (2xO$\underline{C}H_3$), 31.8, 30.4, 29.3, 29.2, 29.1, 28.3 and 22.6 ($(\underline{C}H_2)_7$), 14.0 ($\underline{C}H_3$-chain); HRMS (EI) Calcd for $C_{15}H_{26}O_5$ 286.1780, found 286.1777 $[M]^+$.

***Dimethyl (Z)-2-hydroxymethyl-3-tetradecylbutenedioate* (74).**



To a solution of **77** (2.10 g, 4.46 mmol) in dry MeCN (40 mL) was added BF_3•Et_2O (0.49 mL, 4.01 mmol) at rt and the mixture was stirred for 2 h. The reaction was quenched by adding ice-cold H_2O (30 mL) and further stirred for 5 min. Extraction with CH_2Cl_2 (4x50 mL), drying (Na_2SO_4) and evaporation of solvent *in vacuo* gave 1.69 g of crude product. Further purification by flash chromatography (SiO_2, 3:2/petroleum ether:Et_2O, R_f 0.13) gave **74** (1.56 g, 95%) as a white solid: mp 47-48 °C; IR (CD_2Cl_2 cast) 3506, 2924, 2853, 1726, 1641, 1435, 1316, 1265, 1082 cm^{-1}; ^1H NMR (CD_2Cl_2, 300 MHz) δ 4.36 (d, 2H, J = 6.6 Hz, $C\underline{H}_2OH$), 3.74 (s, 6H, $2xOC\underline{H}_3$), 2.39 (t, 2H, J = 7.5 Hz, $CH_2C\underline{H}_2C{=}C$), 2.11 (t, 1H, J = 6.6 Hz, $CH_2O\underline{H}$), 1.44 (qn, 2H, $C\underline{H}_2CH_2C{=}C$), 1.27 (br s, 22H, $(C\underline{H}_2)_{11}$), 0.88 (t, 3H, J = 6.6 Hz, $C\underline{H}_3$-chain); ^{13}C NMR (CD_2Cl_2, 75 MHz) δ 169.7 and 167.9 ($2x\underline{C}{=}O$), 144.1 and 133.2 ($\underline{C}{=}\underline{C}$), 58.9 ($\underline{C}H_2OH$), 52.5 and 52.4 ($2xO\underline{C}H_3$), 32.3, 30.7, 30.08, 30.05, 30.04, 29.99, 29.86, 29.74, 29.71, 29.67, 28.7 and 23.1 ($(\underline{C}H_2)_{13}$), 14.3 ($\underline{C}H_3$-chain); HRMS (EI) Calcd for $C_{21}H_{38}O_5$ 370.2719, found 370.2715 $[M]^+$; Anal. Calcd for $C_{21}H_{38}O_5$: C, 68.07; H, 10.34. Found: C, 68.11; H, 10.42.

***Dimethyl* (Z)*-2-(2'-trimethylsilylethoxy)methyl-3-octadecylbutenedioate* (75) *and dimethyl* (E)*-2-(2'-trimethylsilylethoxy)methyl-3-octadecylbutenedioate* (76).**



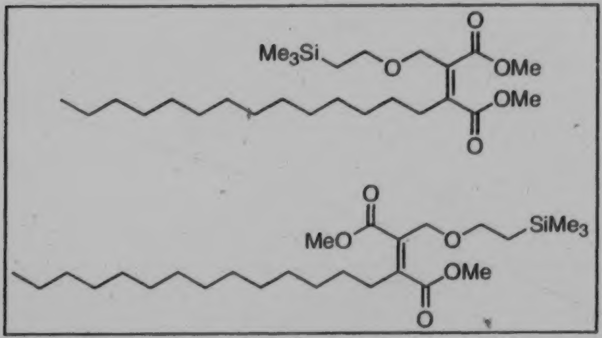
Octylmagnesium chloride (10 mL of a 2.0 M solution in THF, 20 mmol) was added dropwise to a suspension of CuBr•Me$_2$S (4.11 g, 20 mmol) in THF (50 mL) at -40 °C. The resulting yellow suspension was stirred at -40 °C for 2 h, then cooled to -78 °C, and freshly distilled DMAD (2.22 mL, 18 mmol) in THF (10 mL) was added dropwise to give a dark red brown mixture. After 45 min, a HMPA:THF/1:1 solution (20 mL) was added dropwise, and the reaction mixture was stirred for 10 min. Trimethylsilylethoxymethyl chloride (7.1 mL, 40 mmol) in THF (10 mL) was then added dropwise and the reaction mixture was stirred for 17 h at -78 °C. The reaction mixture was warmed to 0 °C, quenched with a saturated aq. NH$_4$Cl solution (50 mL, adjusted to pH 8 with 10% ammonia), and allowed to warm to rt. After 30 min, the mixture was filtered through a pad of Celite®. The aqueous layer was extracted with EtOAc, and the combined organic extracts were washed with a saturated aq. NH$_4$Cl solution, H$_2$O, and brine, dried (Na$_2$SO$_4$), and concentrated *in vacuo*. Further purification by flash chromatography (SiO$_2$, 9:1/hexane:EtOAc, R$_f$**75** 0.39 and R$_f$**76** 0.55 (4:1/hexane:EtOAc)) gave **75** (2.30 g, 33%) and **76** (2.64 g, 38%) as colorless oils.

Data for **75**: IR (CDCl$_3$ cast) 2953, 2926, 2856, 1728, 1640, 1434, 1315, 1262, 1250, 969, 759, 694 cm^{-1}; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 4.20 (s, 2H, OC$\underline{H}_2$C=C), 3.74 and 3.73 (2s, 6H, 2xOC$\underline{H}_3$), 3.51 (dd, 2H, *J* = 9.0, 6.0 Hz, Me$_3$SiCH$_2$C$\underline{H}_2$O), 2.39 (t, 2H, *J* = 7.5 Hz,

$CH_2C\underline{H}_2C{=}C$), 1.47-1.19 (m, 12H, $(C\underline{H}_2)_6$), 0.94-0.82 (m, 5H, $Me_3SiC\underline{H}_2CH_2$ and $C\underline{H}_3$-chain), -0.02 (s, 9H, $(C\underline{H}_3)_3Si$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 169.1 and 167.9 (2x$\underline{C}{=}O$), 142.6 and 133.5 ($\underline{C}{=}\underline{C}$), 68.2 and 65.7 ($\underline{C}H_2O\underline{C}H_2C{=}C$), 52.2 and 51.7 (2x$O\underline{C}H_3$), 31.9, 30.3, 29.5, 29.3, 29.2, 28.3, 22.7 and 18.2 ($(\underline{C}H_2)_7$ and $Me_3Si\underline{C}H_2$), 14.0 ($\underline{C}H_3$-chain), -1.4 ($(\underline{C}H_3)_3Si$); HRMS (ES+) Calcd for $C_{20}H_{38}O_5NaSi$ 409.2386, found 409.2386 $[M+Na]^+$.

Data for **76**: IR ($CHCl_3$ cast) 2953, 2927, 2856, 1730, 1642, 1434, 1247, 1110, 860, 838 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 4.28 (s, 2H, $OC\underline{H}_2C{=}C$), 3.77 and 3.76 (2s, 6H, 2x$OC\underline{H}_3$), 3.49-3.43 (m, 2H, $Me_3SiCH_2C\underline{H}_2O$), 2.39 (t, 2H, J = 7.7 Hz, $CH_2C\underline{H}_2C{=}C$), 1.40 (qn, 2H, $C\underline{H}_2CH_2C{=}C$), 1.23 (br s, 10H, $C\underline{H}_2$-chain), 0.89-0.82 (m, 2H, $Me_3SiC\underline{H}_2CH_2$), 0.85 (t, 3H, J = 6.0 Hz, $C\underline{H}_3$-chain), -0.03 (s, 9H, $(C\underline{H}_3)_3Si$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 168.8 and 167.9 (2x$\underline{C}{=}O$), 139.1 and 135.7 ($\underline{C}{=}\underline{C}$), 67.98 and 67.96 ($\underline{C}H_2O\underline{C}H_2C{=}C$), 51.94 and 51.90 (2x$O\underline{C}H_3$), 31.9, 31.5, 29.34, 29.26, 29.2, 28.5, 22.7 and 18.1 ($(\underline{C}H_2)_7$ and $Me_3Si\underline{C}H_2$), 14.1 ($\underline{C}H_3$-chain), -1.4 ($(\underline{C}H_3)_3Si$); HRMS (EI) Calcd for $C_{20}H_{38}O_5Si$ 386.2488, found 386.2677 $[M]^+$.

***Dimethyl (Z)-2-(2'-trimethylsilylethoxy)methyl-3-tetradecylbutenedioate* (77) *and dimethyl (E)-2-(2'-trimethylsilylethoxy)methyl-3-tetradecylbutenedioate* (78).**



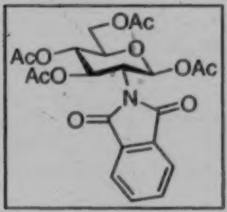
Tetradecylmagnesium chloride (20 mL of a 1.0 M solution in THF, 20 mmol) was added dropwise to a suspension of $CuBr•Me_2S$ (4.11 g, 20 mmol) in THF (50 mL) at -40 °C.

The resulting yellow suspension was stirred at -40 °C for 2 h, then cooled to -78 °C, and freshly distilled DMAD (2.22 mL, 18 mmol) in THF (10 mL) was added dropwise to give a dark red brown mixture. After 1 h, a HMPA:THF/1:1 solution (20 mL) was added dropwise, and the reaction mixture was stirred for 10 min. Trimethylsilylethoxymethyl chloride (7.1 mL, 40 mmol) in THF (10 mL) was then added dropwise and the reaction mixture was stirred for 1 h at -78 °C. The reaction mixture was warmed to -40 °C and stirred for 5.3 h, and then warmed to 0 °C. After 1.5 h, the reaction mixture was quenched with a saturated aq. NH_4Cl solution (50 mL, adjusted to pH 8 with 10% ammonia), and allowed to warm to rt. After 30 min, the mixture was filtered through a pad of Celite®. The aqueous layer was extracted with EtOAc, and the combined organic extracts were washed with a saturated aq. NH_4Cl solution, H_2O, and brine, dried (Na_2SO_4), and concentrated *in vacuo*. Further purification by flash chromatography (SiO_2, 85:15/petroleum ether:Et_2O, R_f**77** 0.52 and R_f**78** 0.47 (3:2/petroleum ether:Et_2O)) gave **77** (2.33 g, 28%) and **78** (4.59 g, 54%) as colorless oils.

Data for **77**: IR ($CHCl_3$ cast) 2924, 2853, 1728, 1633, 1458, 1434, 1262, 1250, 1079, 859, 837 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 4.20 (s, 2H, $OC\underline{H}_2C{=}C$), 3.74 and 3.73 (2s, 6H, $2xOC\underline{H}_3$), 3.52 (dd, 2H, J = 9.2, 8.1 Hz, $Me_3SiCH_2C\underline{H}_2O$), 2.39 (t, 2H, J = 7.3 Hz, $CH_2C\underline{H}_2C{=}C$), 1.47-1.20 (m, 24H, $(C\underline{H}_2)_{12}$), 0.94-0.85 (m, 5H, $Me_3SiC\underline{H}_2CH_2$ and $C\underline{H}_3$-chain), -0.01 (s, 9H, $(C\underline{H}_3)_3Si$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 169.1 and 168.0 ($2x\underline{C}{=}O$), 142.7 and 133.5 ($\underline{C}{=}\underline{C}$), 68.3 and 65.7 ($\underline{C}H_2O\underline{C}H_2C{=}C$), 52.2 and 51.9 ($2xO\underline{C}H_3$), 32.0, 30.2, 29.7, 29.54, 29.45, 29.40, 28.3, 22.7 and 18.1 ($(\underline{C}H_2)_{13}$ and $Me_3Si\underline{C}H_2$), 14.1 ($\underline{C}H_3$-chain), -1.4 ($((\underline{C}H_3)_3Si)$); HRMS (EI) Calcd for $C_{26}H_{50}O_5Si$ 470.3427, found 470.3428 $[M]^+$; Anal. Calcd for $C_{26}H_{50}O_5Si$: C, 66.34; H, 10.71. Found: C, 66.63; H, 10.80.

Data for **78**: IR ($CHCl_3$ cast) 2952, 2925, 2854, 1732, 1433 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 4.28 (s, 2H, $OC\underline{H}_2C{=}C$), 3.77 and 3.76 (2s, 6H, $2xOC\underline{H}_3$), 3.47 (dd, 2H, J = 8.7, 8.1 Hz, $Me_3SiCH_2C\underline{H}_2O$), 2.39 (t, 2H, J = 7.5 Hz, $CH_2C\underline{H}_2C{=}C$), 1.40 (qn, 2H, $C\underline{H}_2CH_2C{=}C$), 1.29-1.20 (m, 22H, $(C\underline{H}_2)_{11}$), 0.90-0.85 (m, 5H, $Me_3SiC\underline{H}_2CH_2$ and $C\underline{H}_3$-chain), -0.02 (s, 9H, $(C\underline{H}_3)_3Si$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 168.5 and 167.6 ($2x\underline{C}{=}O$), 138.9 and 133.5 ($\underline{C}{=}\underline{C}$), 67.8 ($\underline{C}H_2O\underline{C}H_2C{=}C$), 51.6 and 51.5 ($2xO\underline{C}H_3$), 31.8, 31.3, 29.5, 29.3, 29.2, 29.1, 28.3, 22.5 and 17.8 ($(\underline{C}H_2)_{13}$ and $Me_3Si\underline{C}H_2$), 13.9 ($\underline{C}H_3$-chain), -1.6 ($(\underline{C}H_3)_3Si$); HRMS (EI) Calcd for $C_{20}H_{38}O_5Si$ 470.3427, found 470.3422 $[M]^+$; Anal. Calcd for $C_{26}H_{50}O_5Si$: C, 66.34; H, 10.71. Found: C, 66.65; H, 10.95.

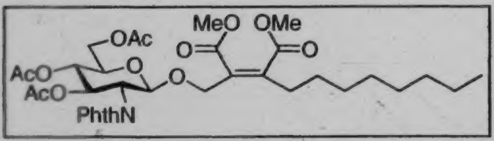
***1,3,4,6-Tetra-*O*-acetyl-2-deoxy-2-phthalimido-β-*D*-glucopyranoside* (79).**



The known compound **79**[401-409] was prepared using the following procedure. A mixture of *D*-glucosamine hydrochloride (**53**) (10 g, 46.37 mmol), Na_2CO_3 (5.8 g, 46.37 mmol), phthalic anhydride (6.87 g, 46.37 mmol), and H_2O (60 mL) was stirred at rt for 6 h. The H_2O was removed under reduced pressure and the residue was dissolved in pyridine (150 mL) and Ac_2O (75 mL), and a catalytic amount of DMAP was added. The reaction mixture was stirred at rt for 13 h. Most of the solvent was removed under reduced pressure. The reaction mixture was then dissolved in CH_2Cl_2 (150 mL), washed with a 6 N HCl solution, a saturated aq. $NaHCO_3$ solution, H_2O, and brine, dried (Na_2SO_4), and concentrated *in vacuo*. Further purification by flash chromatography (SiO_2; 1:1/hexane:EtOAc, R_f 0.27) gave **79** (10.69 g, 48%) as a white solid: mp 69-71 °C (lit.[410]

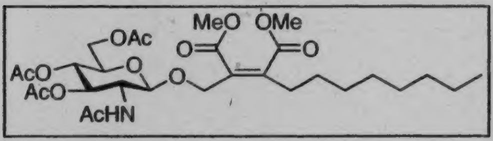
mp 77-78 °C); $[\alpha]^{26}_{D}$ +85° (*c* 0.34, $CHCl_3$) (lit.[409] $[\alpha]^{20}_{D}$ +55.8° (*c* 1.0, CH_2Cl_2)) (lit.[408] $[\alpha]^{22}_{D}$ +62° (*c* 0.56, $CHCl_3$)) (lit.[407] $[\alpha]^{22}_{D}$ +64° (*c* 1.2, $CHCl_3$)) (lit.[410] $[\alpha]^{25}_{D}$ +68° (*c* 0.5, $CHCl_3$)) (lit.[411] $[\alpha]^{20}_{D}$ +59.1° (*c* 1.3, $CHCl_3$)) (lit.[401] $[\alpha]^{15}_{D}$ +37° (*c* 7, $CHCl_3$)); IR ($CHCl_3$ cast) (lit.[408]) 2950, 1753, 1720, 1469, 1431, 1385, 1216, 1080, 1046, 972, 906, 723 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) (lit.[406-410,412]) δ 7.85 (dd, 2H, J_{ortho} = 5.4 Hz, J_{meta} = 3.0 Hz, C$\underline{H}$-2'), 7.73 (dd, 2H, J_{ortho} = 5.4 Hz, J_{meta} = 3.0 Hz, C$\underline{H}$-1'), 6.50 (d, 1H, $J_{1,2}$ = 9.0 Hz, C$\underline{H}$-1), 5.87 (dd, 1H, $J_{3,2}$ = 10.8 Hz, $J_{3,4}$ = 9.3 Hz, C$\underline{H}$-3), 5.20 (dd, 1H, $J_{4,5}$ = 10.5 Hz, $J_{4,3}$ = 9.3 Hz, C$\underline{H}$-4), 4.46 (dd, 1H, $J_{2,3}$ = 10.8 Hz, $J_{2,1}$ = 9.0 Hz, C$\underline{H}$-2), 4.35 (dd, 1H, $J_{6,6'}$ = 12.6 Hz, $J_{6,5}$ = 4.5 Hz, C$\underline{H}$-6), 4.13 (dd, 1H, $J_{6',6}$ = 12.6 Hz, $J_{6',5}$ = 2.1 Hz, C$\underline{H}$-6'), 4.01 (ddd, $J_{5,4}$ = 10.5 Hz, $J_{5,6}$ = 4.5 Hz, $J_{5,6'}$ = 2.1 Hz, C$\underline{H}$-5), 2.10, 2.02, 1.98 and 1.85 (4s, 12H, 4xC=OC$\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) (lit.[406-408]) δ 170.7, 170.1, 169.5, 168.7 and 167.4 (6x$\underline{C}$=O), 134.5 ($\underline{C}$-1'), 123.8 ($\underline{C}$-2'), 89.8 ($\underline{C}$-1), 72.7 ($\underline{C}$-5), 70.6 ($\underline{C}$-3), 68.4 ($\underline{C}$-4), 60.4 ($\underline{C}$-6), 53.6 ($\underline{C}$-2), 20.83, 20.80, 20.7 and 20.5 (4xC=O$\underline{C}H_3$); MS (ES+) Calcd for $C_{22}H_{23}NO_{11}$ 477.42, found 500.1 $[M+Na]^+$; Anal. Calcd for $C_{22}H_{23}NO_{11}$: C, 55.35; H, 4.86; N, 2.93. Found: C, 55.42; H, 4.88; N, 2.74.

O-*(Dimethyl (Z)-2-oxymethyl-3-octylbutenedioate) 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-1-β-*D*-glucopyranoside* (80).



This glycosylation was done using the method described by Füjedi.[185] To a mixture of alcohol **73** (112 mg, 0.39 mmol) and thioglycoside **72** (217 mg, 0.41 mmol) in CH_2Cl_2 (14 mL) in the presence of 4 Å molecular sieves was added DMTST (151 mg, 0.59 mmol). After 4.5 h the reaction mixture was diluted with CH_2Cl_2, filtered, and concentrated *in vacuo*. Further purification by flash chromatography (SiO_2, 3:2/hexane:EtOAc, R_f 0.18) gave **80** (222 mg, 81%) as a colorless oil: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.82 (dd, 2H, J = 5.4 Hz, J = 3.0 Hz, C$\underline{\text{H}}$-3 and C$\underline{\text{H}}$-6 of Phth), 7.70 (dd, 2H, J = 5.4 Hz, J = 3.0 Hz, C$\underline{\text{H}}$-4 and C$\underline{\text{H}}$-5 of Phth), 5.77 (dd, 1H, $J_{3,2}$ = 10.8 Hz, $J_{3,4}$ = 9.0 Hz, C$\underline{\text{H}}$-3), 5.40 (d, 1H, $J_{1,2}$ = 8.4 Hz, C$\underline{\text{H}}$-1), 5.15 (dd, 1H, $J_{4,5}$ = 10.2 Hz, $J_{4,3}$ = 9.0 Hz, C$\underline{\text{H}}$-4), 4.53 (d, 1H, $J_{1',1''}$ = 12.0 Hz, C$\underline{\text{H}}$-1'), 4.32 (d, 1H, $J_{1'',1'}$ = 12.0 Hz, C$\underline{\text{H}}$-1"), 4.32 (dd, 1H, $J_{6,6'}$ = 12.3 Hz, $J_{6,5}$ = 4.5 Hz, C$\underline{\text{H}}$-6), 4.27 (dd, 1H, $J_{2,3}$ = 10.2 Hz, $J_{2,1}$ = 8.4 Hz, C$\underline{\text{H}}$-2), 4.16 (dd, 1H, $J_{6',6}$ = 12.3 Hz, $J_{6',5}$ = 2.4 Hz, C$\underline{\text{H}}$-6'), 3.85 (ddd, $J_{5,4}$ = 10.2 Hz, $J_{5,6}$ = 4.5 Hz, $J_{5,6'}$ = 2.4 Hz, C$\underline{\text{H}}$-5), 3.67 and 3.36 (2s, 6H, 2xOC$\underline{\text{H}}_3$), 2.28 (t, 2H, $J_{4',5'}$ = 7.8 Hz, C$\underline{\text{H}}_2$-4'), 2.10, 2.01 and 1.82 (3s, 9H, 3xC=OC$\underline{\text{H}}_3$), 1.34-1.22 (m, 2H, C$\underline{\text{H}}_2$-5'), 1.20 (br s, 10H, (C$\underline{\text{H}}_2$)$_5$), 0.86 (t, 3H, J = 6.9 Hz, C$\underline{\text{H}}_3$-chain); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 170.7, 170.2 and 169.5 (3x$\underline{\text{C}}$=OCH$_3$), 169.1 and 166.5 (2xN$\underline{\text{C}}$=O), 146.4 (OC$\underline{\text{C}}$=$\underline{\text{C}}$CO of Phth), 134.2 and 123.6 (H$\underline{\text{C}}$=$\underline{\text{C}}$H of Phth), 131.6 and 129.4 ($\underline{\text{C}}$=$\underline{\text{C}}$), 97.6 ($\underline{\text{C}}$-1), 72.0 ($\underline{\text{C}}$-5), 70.7 ($\underline{\text{C}}$-3), 69.0 ($\underline{\text{C}}$-4), 64.3 ($\underline{\text{C}}$-1'), 62.0 ($\underline{\text{C}}$-6), 54.5 ($\underline{\text{C}}$-2), 52.2 and 51.9 (2xO$\underline{\text{C}}$H$_3$), 31.8, 30.6, 29.32, 29.25, 29.1, 28.0 and 22.7 (7x$\underline{\text{C}}$H$_2$-chain), 20.8, 20.7 and 20.5 (3xC=O$\underline{\text{C}}$H$_3$), 14.1 ($\underline{\text{C}}$H$_3$-chain).

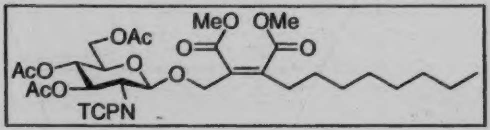
O-*(Dimethyl (Z)-2-oxymethyl-3-octylbutenedioate) 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-*D*-glucopyranoside* (81).



The transformation of the tetrachlorophthalimido moiety into an acetamido group was performed using a method described by Debenham.[205] To compound **82** (4.7 mg, 5.6 μmol) in a 2:1:1/MeCN:EtOH:THF mixture of solvents (0.4 mL) was added ethylenediamine (2 μL) before heating to 60 °C. The reaction mixture was stirred for 2 h and then allowed to cool to rt before being concentrated under reduced pressure and filtered through a short column of SiO_2 (1:9/MeOH:CH_2Cl_2). The residue was treated with Et_3N (0.2 mL) in EtOH (0.4 mL) and acetic anhydride at rt overnight. The reaction mixture was concentrated *in vacuo*. Further purification by reversed-phase HPLC (C_{18} Bondpak, flow rate: 15 mL/min, gradient elution: 65-80% MeCN in H_2O over 6 min, 80-100% MeCN in H_2O over 11 min, 100% MeCN for 6 min, 100-65% MeCN in H_2O over 3 min) gave **81** (t_R 7.9 min, 2.4 mg, 70%) as a white film: $[\alpha]_D^{26}$ -55° (*c* 0.08, $CHCl_3$); IR ($CHCl_3$ cast) 3282, 2954, 2927, 2856, 1749, 1660, 1552, 1435, 1368, 1240, 1046 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 5.49 (d, 1H, $J_{NH,2}$ = 8.7 Hz, N$\underline{H}$), 5.17-5.03 (m, 2H, C$\underline{H}$-3 and C$\underline{H}$-4), 4.57 (d, 1H, $J_{1,2}$ = 8.4 Hz, C$\underline{H}$-1), 4.50 (d, 2H, J = 3.3 Hz, OC$\underline{H}_2$C=C), 4.24 (dd, 1H, $J_{6,6'}$ = 12.3 Hz, $J_{6,5}$ = 4.5 Hz, C$\underline{H}$-6), 4.12 (dd, 1H, $J_{6',6}$ = 12.3 Hz, $J_{6',5}$ = 2.7 Hz, C$\underline{H}$-6'), 3.95 (dd, 1H, $J_{2,NH}$ = 8.7 Hz, $J_{2,1}$ = 8.4 Hz, C$\underline{H}$-2), 3.76 and 3.74 (2s, 6H, 2xOC$\underline{H}_3$), 3.67-3.61 (m, 1H, C$\underline{H}$-5), 2.46-2.28 (m, 2H, CH_2C$\underline{H}_2$C=C), 2.07 (s, 3H, C=OC$\underline{H}_3$), 2.00 (s, 6H, C=OC$\underline{H}_3$), 1.91 (s, 3H, C=OC$\underline{H}_3$), 1.43-1.32 (m, 2H, C$\underline{H}_2$$CH_2$C=C), 1.24 (br s, 10H, (C$\underline{H}_2$)$_5$), 0.86 (t, 3H, J = 6.9 Hz, C$\underline{H}_3$-chain); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 202.9, 172.5, 171.0, 169.4 (6x$\underline{C}$=O), 143.7 and 132.4 ($\underline{C}$=$\underline{C}$), 99.0 ($\underline{C}$-1), 72.9 ($\underline{C}$-3), 72.2 ($\underline{C}$-5), 68.4 ($\underline{C}$-4), 63.9 ($\underline{C}$-1'), 62.1 ($\underline{C}$-6), 54.1 ($\underline{C}$-2), 52.4 (2xO$\underline{C}H_3$), 31.8, 29.9, 29.3, 29.2 and 28.5 (7x$\underline{C}H_2$-chain), 23.0, 20.8 and 20.7 (3xC=O$\underline{C}H_3$), 14.1 ($\underline{C}H_3$-chain); HRMS (ES+) Calcd for $C_{29}H_{45}NO_{13}K$ 654.2523, found

654.2513 [M+K]$^+$, Calcd for $C_{29}H_{45}NO_{13}Na$ 638.2783, found 638.2769 [M+Na]$^+$, Calcd for $C_{29}H_{46}NO_{13}$ 616.2963, found 616.2965 [M+H]$^+$.

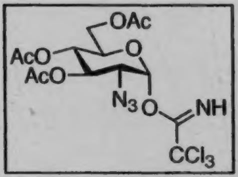
O-(Dimethyl (Z)-2-oxymethyl-3-octylbutenedioate) 3,4,6-tri-O-acetyl-2-deoxy-2-tetrachlorophthalimido-1-β-D-glucopyranoside **(82).**



This glycosylation was done using the method described by Füjedi.[185] To a mixture of alcohol **73** (44 mg, 0.16 mmol) and thioglycoside **52** (123 mg, 0.19 mmol) in CH_2Cl_2 (9 mL) in the presence of 4 Å molecular sieves was added DMTST (110 mg, 0.43 mmol). After 4.5 h the reaction mixture was diluted with CH_2Cl_2, filtered, and concentrated *in vacuo*. Further purification by reversed-phase HPLC (C_{18} Bondpak, flow rate: 15 mL/min, gradient elution: 65-80% MeCN in H_2O over 6 min, 80-100% MeCN in H_2O over 11 min, 100% MeCN for 6 min, 100-65% MeCN in H_2O over 3 min) gave **82** (t_R 11.7 min, 91 mg, 70%) as a white solid: IR ($CHCl_3$ cast) 3491, 3024, 2953, 2927, 2856, 1783, 1727, 1643, 1435, 1391, 1371, 1330, 1229, 1166, 1113, 1048, 908, 754, 740 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.71 (dd, 1H, $J_{3,2}$ = 10.2 Hz, $J_{3,4}$ = 9.0 Hz, C$\underline{H}$-3), 5.36 (d, 1H, $J_{1,2}$ = 8.4 Hz, C$\underline{H}$-1), 5.16 (dd, 1H, $J_{4,5}$ = 10.5 Hz, $J_{4,3}$ = 9.0 Hz, C$\underline{H}$-4), 4.56 (d, 1H, $J_{1',1''}$ = 12.0 Hz, C$\underline{H}$-1'), 4.32 (d, 1H, $J_{1'',1'}$ = 12.0 Hz, C$\underline{H}$-1"), 4.32 (dd, 1H, $J_{6,6'}$ = 12.3 Hz, $J_{6,5}$ = 4.5 Hz, C$\underline{H}$-6), 4.24 (dd, 1H, $J_{2,3}$ = 10.2 Hz, $J_{2,1}$ = 8.4 Hz, C$\underline{H}$-2), 4.16 (dd, 1H, $J_{6',6}$ = 12.3 Hz, $J_{6',5}$ = 2.4 Hz, C$\underline{H}$-6'), 3.82 (ddd, $J_{5,4}$ = 10.5 Hz, $J_{5,6}$ = 4.5 Hz, $J_{5,6'}$ = 2.4 Hz, C$\underline{H}$-5), 3.66 and 3.55 (2s, 6H, 2xOC$\underline{H}_3$), 2.32-2.26 (m, 2H, C$\underline{H}_2$-4'), 2.10, 2.01 and 1.87 (3s, 9H, 3xC=OC$\underline{H}_3$), 1.34-1.24 (m, 2H, C$\underline{H}_2$-5'), 1.21 (br s, 10H, (C$\underline{H}_2)_5$), 0.86 (t, 3H, J =

6.9 Hz, $C\underline{H}_3$-chain); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 170.7, 170.5 and 169.4 (3x$\underline{C}$=OCH_3), 168.8 and 168.6 (2xN$\underline{C}$=O), 145.4 (OC$\underline{C}$=$\underline{C}$CO of TCP), 130.3 and 121.2 (H$\underline{C}$=$\underline{C}$H of TCP), 130.0 and 127.2 ($\underline{C}$=$\underline{C}$), 97.7 ($\underline{C}$-1), 72.0 ($\underline{C}$-5), 70.8 ($\underline{C}$-3), 68.6 ($\underline{C}$-4), 65.0 ($\underline{C}$-1'), 61.9 ($\underline{C}$-6), 55.3 ($\underline{C}$-2), 52.3 and 52.2 (2xO$\underline{C}H_3$), 31.9, 30.5, 29.4, 29.3, 29.2, 28.1 and 22.7 (7x$\underline{C}H_2$-chain), 20.8, 20.7 and 20.6 (3xC=O$\underline{C}H_3$), 14.1 ($\underline{C}H_3$-chain); HRMS (ES+) Calcd for $C_{35}H_{41}NCl_4O_{14}Na$ 862.1179, found 862.1168 $[M+Na]^+$.

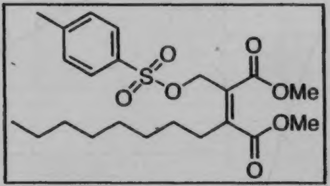
***3,4,6-Tri-O-acetyl-2-azido-2-deoxy-α-D-glucopyranosyltrichloroacetimidate* (85).**



The known compound **85**[413-415] was prepared using the following imidation method. To a solution of alcohol **93** (791 mg, 2.39 mmol) in dry CH_2Cl_2 (13 mL) was added freshly distilled trichloroacetonitrile (4.8 mL, 47.8 mmol) and DBU (0.18 mL, 1.20 mmol) at 5 °C. After being stirred for 1 h the reaction mixture was concentrated *in vacuo* (<30 °C) and the residue obtained was purified by flash chromatography (SiO_2; 5:3/hexane:EtOAc, R_f 0.36) to give **85** (827 mg, 73%) as a white solid: mp 130 °C (lit.[415,416] mp 130-131 °C) (lit.[413] mp 130 °C); $[\alpha]_D^{26}$ +118° (*c* 0.33, $CHCl_3$) (lit.[414] $[\alpha]_D^{25}$ +119° (*c* 1.00, $CHCl_3$)); IR ($CDCl_3$ cast) (lit.[413,414]) 3321, 2961, 2114, 1752, 1678, 1433, 1368, 1226, 1052, 1020 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) (lit.[414]) δ 8.81 (s, 1H, C=N$\underline{H}$), 6.47 (d, 1H, $J_{1,2}$ = 3.9 Hz, C$\underline{H}$-1), 5.49 (dd, 1H, $J_{3,2}$ = 10.5 Hz, $J_{3,4}$ = 9.3 Hz, C$\underline{H}$-3), 5.12 (t, 1H, $J_{4,3}$ = 9.3 Hz, C$\underline{H}$-4), 4.25 (dd, 1H, $J_{6,6'}$ = 12.0 Hz, $J_{6,5}$ = 4.5 Hz, C$\underline{H}$-6), 4.19 (ddd, 1H, $J_{5,4}$ = 10.2 Hz, $J_{5,6}$ = 4.5 Hz, $J_{5,6'}$ = 1.8 Hz, C$\underline{H}$-5), 4.07 (dd, 1H, $J_{6',6}$ = 12.0 Hz, $J_{6',5}$ = 1.8 Hz, C$\underline{H}$-6'), 3.75 (dd, 1H, $J_{2,3}$ = 10.5 Hz, $J_{2,1}$ = 3.9 Hz, C$\underline{H}$-2), 2.08, 2.03 and 2.02 (3s, 9H, 3xC=OC$\underline{H}_3$); ^{13}C NMR

($CDCl_3$, 75 MHz) δ 170.5, 169.8 and 169.6 (3xC=O), 160.5 (C=NH), 94.0 (C-1), 90.5 (CCl_3), 70.7 (C-3), 70.1 (C-5), 68.0 (C-4), 61.4 (C-6), 60.7 (C-2), 20.65, 20.64 and 20.56 (3xC=OCH_3); HRMS (ES+) Calcd for $C_{14}H_{17}Cl_3N_4O_8Na$ 497.0010, found 497.0024 $[M+Na]^+$; Anal. Calcd for $C_{14}H_{17}Cl_3N_4O_8$: C, 35.35; H, 3.60; N, 11.78. Found: C, 34.96; H, 3.13; N, 11.49.

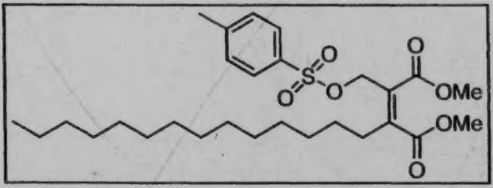
***Dimethyl (Z)-2-p-toluenesulfonylmethyl-3-octylbutenedioate* (86).**



Alcohol **73** (90 mg, 0.31 mmol) was dissolved in CH_2Cl_2 (6 mL) and cooled at 0 °C. Et_3N (0.17 mL, 1.25 mmol), DMAP (39 mg, 0.31 mmol), and *p*-TsCl (90 mg, 0.31 mmol) were added successively. The reaction mixture was stirred at 0 °C for 3.5 h. Et_2O and H_2O were added, the organic layer was washed successively with 1 M aq. HCl, 5% aq. $NaHCO_3$, and H_2O, dried (Na_2SO_4) and concentrated *in vacuo*. Further purification by flash chromatography (SiO_2, 1:1/petroleum ether:Et_2O, R_f 0.30) gave **86** (101 mg, 72%) as a white solid: mp 40-41 °C; IR ($CDCl_3$ cast) 3026, 2928, 2856, 1729, 1436, 1320, 1216, 1096, 756 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.77 (d, 2H, *J* = 8.1 Hz, $SO_2C=CH$ of Ts), 7.32 (d, 2H, *J* = 8.1 Hz, MeC=CH of Ts), 4.78 (s, 2H, $OCH_2C=C$), 3.74 and 3.59 (2s, 6H, $2xOCH_3$), 2.42 (s, 3H, CH_3 of Ts), 2.33-2.27 (m, 2H, $CH_2CH_2C=C$), 1.40-1.30 (m, 2H, $CH_2CH_2C=C$), 1.21 (br s, 10H, $(CH_2)_5$), 0.85 (t, 3H, *J* = 6.9 Hz, CH_3-chain); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 169.0 and 165.1 (2xC=O), 151.8 ($C=CSO_2$), 145.0 and 132.9 (C=C), 129.8 and 128.1 (HC=CH of Ts), 124.4 (MeC=C of Ts), 64.2 (CH_2OTs), 52.4

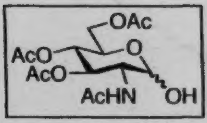
(2xO$\underline{C}H_3$), 31.8, 31.3, 29.3, 29.2, 29.1, 27.7 and 22.6 ($(\underline{C}H_2)_7$), 21.7 ($\underline{C}H_3$ of Ts), 14.1 ($\underline{C}H_3$-chain); HRMS (EI) Calcd for $C_{22}H_{32}O_7S$ 440.1869, found 440.1870 $[M]^+$.

***Dimethyl (Z)-2-*p*-toluenesulfonylmethyl-3-tetradecylbutenedioate* (87).**



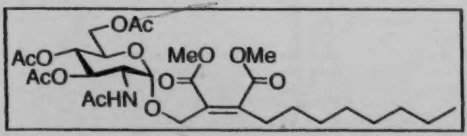
This tosylation was performed using an adaptation of the method described by Kabalka.[417] Alcohol **74** (30 mg, 81 μmol) was dissolved in $CHCl_3$ (1 mL) and cooled at 0 °C. Pyridine (13 μL, 162 μmol) and *p*-TsCl (23 mg, 122 μmol) were added successively. The reaction mixture was stirred at rt for 21 h. Et_2O (10 mL) and H_2O (1 mL) were added, the organic layer was washed successively with 1 M aq. HCl, 5% aq. $NaHCO_3$, and H_2O, dried (Na_2SO_4) and concentrated *in vacuo*. Further purification by flash chromatography (SiO_2, 1:1/petroleum ether:Et_2O, R_f 0.37) gave **87** (27 mg, 63%) as a colorless oil: IR ($CDCl_3$ cast) 2925, 2854, 1732, 1643, 1435, 1319, 1270, 1189, 1177, 1096, 946, 815, 771 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.78 (d, 2H, J = 8.4 Hz, $SO_2C{=}C\underline{H}$ of Ts), 7.33 (d, 2H, J = 8.4 Hz, $MeC{=}C\underline{H}$ of Ts), 4.79 (s, 2H, $OC\underline{H}_2C{=}C$), 3.76 and 3.61 (2s, 6H, 2x$OC\underline{H}_3$), 2.44 (s, 3H, $C\underline{H}_3$ of Ts), 2.34-2.29 (m, 2H, $CH_2C\underline{H}_2C{=}C$), 1.39-1.24 (m, 2H, $C\underline{H}_2CH_2C{=}C$), 1.24 (br s, 22H, $(C\underline{H}_2)_{11}$), 0.86 (t, 3H, J = 7.2 Hz, $C\underline{H}_3$-chain); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 169.1 and 165.2 (2x$\underline{C}{=}O$), 151.9 ($C{=}\underline{C}SO_2$), 145.0 and 132.9 ($\underline{C}{=}\underline{C}$), 129.9 and 128.1 ($H\underline{C}{=}\underline{C}H$ of Ts), 124.5 ($Me\underline{C}{=}C$ of Ts), 64.2 ($\underline{C}H_2OTs$), 52.5 and 52.4 (2x$O\underline{C}H_3$), 32.0, 31.4, 29.7, 29.6, 29.5, 29.41, 29.37, 29.30, 27.8 and 22.7 ($(\underline{C}H_2)_{13}$), 21.7 ($\underline{C}H_3$ of Ts), 14.2 ($\underline{C}H_3$-chain); HRMS (EI) Calcd for $C_{28}H_{44}O_7S$ 524.2808, found 524.2809 $[M]^+$.

3,4,6-Tri-**O*****-acetyl-2-acetamido-2-deoxy-*****D*****-glucopyranoside* (89).**



The known compound **89**[209,212-214,418-425] was prepared using an adaptation of the method described by Excoffier.[215] To a solution of 1,3,4,6-tetra-*O*-acetyl-2-acetamido-2-deoxy-β-*D*-glycopyranose (**88**) (200 mg, 0.51 mmol) in DMF (1.5 mL) at 50 °C was added hydrazine acetate (56 mg, 0.61 mmol). The reaction mixture was stirred at 50 °C until complete dissolution (~3 min). After being stirred for 2 h at rt, the reaction mixture was diluted with EtOAc (20 mL), washed with brine (2x5 mL), dried (Na_2SO_4), filtered, and concentrated under reduced pressure. Further purification by flash chromatography (SiO_2, 98:2/$CHCl_3$:MeOH, R_f 0.28 (95:5/$CHCl_3$:MeOH)) gave **89** (145 mg, 82%) as a colorless oil: $[\alpha]_D^{26}$ +43° (*c* 1.0, $CHCl_3$) (lit.[418] $[\alpha]_D^{23}$ +52.2° (*c* 1.01, $CHCl_3$)); IR ($CHCl_3$ cast) (lit.[418,425]) 3361, 3023, 2959, 1747, 1539, 1435, 1369, 1237, 1046 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) (lit.[418]) δ 5.93 (d, 1H, $J_{NH,2}$ = 9.6 Hz, N$\underline{H}$ of α-anomer), 5.27 (dd, 1H, $J_{3,2}$ = 10.8 Hz, $J_{3,4}$ = 9.6 Hz, C$\underline{H}$-3), 5.23 (d, 1H, $J_{1,2}$ = 3.0 Hz, C$\underline{H}$-1 of α-anomer), 5.10 (t, 1H, $J_{4,3}$ = 9.6 Hz, C$\underline{H}$-4), 4.62 (d, 1H, $J_{1,2}$ = 8.1 Hz, C$\underline{H}$-1 of β-anomer), 4.32-4.07 (m, 4H, C$\underline{H}$-2, C$\underline{H}$-5 and C$\underline{H}_2$-6), 2.06, 2.01 and 2.00 (3s, 9H, 3xC=OC$\underline{H}_3$), 1.93 (s, 3H, NHCOC$\underline{H}_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) (lit.[404,418]) δ 171.4, 171.0, 170.8 and 169.5 (4x$\underline{C}$=O), 91.4 ($\underline{C}$-1 of α-anomer), 71.0 ($\underline{C}$-5), 68.3 ($\underline{C}$-3), 67.4 ($\underline{C}$-4), 62.2 ($\underline{C}$-6), 52.4 ($\underline{C}$-2), 23.0 (NHCO$\underline{C}H_3$), 20.73, 20.70 and 20.6 (3xC=O$\underline{C}H_3$); HRMS (ES+) Calcd for $C_{14}H_{21}NO_9Na$ 370.1114, found 370.1107 $[M+Na]^+$.

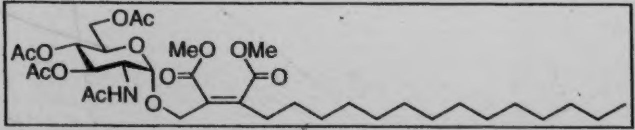
O-*(Dimethyl (Z)-2-oxymethyl-3-octylbutenedioate) 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-*D*-glucopyranoside* (90).



To a stirred solution of $SnCl_2$ (16 mg, 0.08 mmol) in dry MeCN (0.5 mL) was added consecutively thiophenol (34 μL, 0.34 mmol), Et_3N (35 μL, 0.25 mmol), and azide **94** (34 mg, 0.06 mmol) in dry MeCN (2 mL). The reaction mixture was stirred at rt for 20 min, after which time it was diluted with CH_2Cl_2, and washed with 2 N NaOH. The aqueous phase was extracted with CH_2Cl_2, and the combined organic extracts were dried (Na_2SO_4), and concentrated under reduced pressure. The residue was dissolved in pyridine (0.84 mL) and Ac_2O (0.33 mL) and stirred at rt for 17 h. The solution was evaporated to dryness, and then coevaporated with toluene. Further purification by flash chromatography (SiO_2; 1:3/hexane:EtOAc, R_f 0.21) gave **90** (18 mg, 54%) as a white solid: $[\alpha]_D^{26}$ +173° (*c* 0.08, $CHCl_3$); IR ($CHCl_3$ cast) 3363, 2953, 2928, 2856, 1746, 1684, 1537, 1435, 1367, 1232, 1047 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 5.73 (d, 1H, $J_{NH,2}$ = 9.6 Hz, N$\underline{H}$), 5.15-5.05 (m, 2H, C$\underline{H}$-3 and C$\underline{H}$-4), 4.84 (d, 1H, $J_{1,2}$ = 3.6 Hz, C$\underline{H}$-1), 4.49 (d, 1H, $J_{6,6'}$ = 12.0 Hz, C$\underline{H}$-6), 4.35-4.27 (m, 1H, C$\underline{H}$-2), 4.24 (d, 1H, $J_{6',6}$ = 12.0 Hz, C$\underline{H}$-6'), 4.21 (dd, 1H, $J_{1',1''}$ = 12.6 Hz, $J_{1',1}$ = 4.2 Hz, C$\underline{H}$-1'), 4.08 (dd, 1H, $J_{1'',1'}$ = 12.6 Hz, $J_{1'',1}$ = 2.4 Hz, C$\underline{H}$-1"), 3.97-3.91 (m, 1H, C$\underline{H}$-5), 3.77 and 3.76 (2s, 6H, 2xOC$\underline{H}_3$), 2.38 (t, 2H, $J_{4',5'}$ = 7.2 Hz, C$\underline{H}_2$-4'), 2.07, 1.99, 1.98 and 1.92 (4s, 12H, 4xC=OC$\underline{H}_3$), 1.47-1.35 (m, 2H, C$\underline{H}_2$-5'), 1.23 (br s, 10H, (C$\underline{H}_2)_5$), 0.84 (t, 3H, *J* = 6.6 Hz, C$\underline{H}_3$-chain); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 171.2, 170.6, 170.1, 169.3, 168.7 and 167.1 (6x$\underline{C}$=O), 144.8 and 130.4 ($\underline{C}$=$\underline{C}$), 97.5 ($\underline{C}$-1), 71.2 ($\underline{C}$-3), 68.4 ($\underline{C}$-5), 68.0 ($\underline{C}$-4), 63.7 ($\underline{C}$-6), 61.9 ($\underline{C}$-1'), 52.5 and 52.4

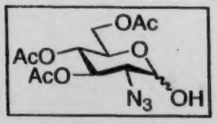
(2xO$\underline{C}H_3$), 51.7 ($\underline{C}$-2), 31.8, 30.5, 29.4, 29.3, 29.1, 28.4 and 22.6 (7x$\underline{C}H_2$-chain), 23.1, 20.7 and 20.6 (4xC=O$\underline{C}H_3$), 14.1 ($\underline{C}H_3$-chain); HRMS (ES+) Calcd for $C_{29}H_{45}NO_{13}Na$ 638.2789, found 638.2796 [M+Na]$^+$.

***O-(Dimethyl (Z)-2-oxymethyl-3-tetradecylbutenedioate) 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-glucopyranoside* (91).**



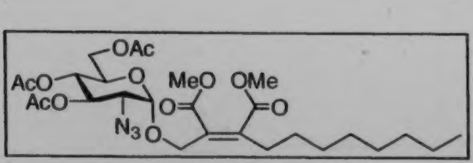
The reaction of $SnCl_2$ (26 mg, 0.14 mmol), thiophenol (56 μL, 0.55 mmol), Et_3N (57 μL, 0.41 mmol), and azide **95** (63 mg, 0.09 mmol) was performed as described for the synthesis of **90**. Further purification by flash chromatography (SiO_2; 1:1/hexane:EtOAc, R_f 0.12 or R_f 0.28 (1:3/hexane:EtOAc)) gave **91** (40 mg, 63%) as a white solid: $[\alpha]_D^{26}$ +59° (*c* 3.46, $CHCl_3$); IR ($CHCl_3$ cast) 3362, 2925, 2854, 1747, 1685, 1537, 1435, 1367, 1233, 1047 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.73 (d, 1H, $J_{NH,2}$ = 9.6 Hz, N$\underline{H}$), 5.15-5.05 (m, 2H, C$\underline{H}$-3 and C$\underline{H}$-4), 4.84 (d, 1H, $J_{1,2}$ = 3.6 Hz, C$\underline{H}$-1), 4.49 (d, 1H, $J_{6,6'}$ = 12.0 Hz, C$\underline{H}$-6), 4.35-4.27 (m, 1H, C$\underline{H}$-2), 4.24 (d, 1H, $J_{6',6}$ = 12.0 Hz, C$\underline{H}$-6'), 4.21 (dd, 1H, $J_{1',1''}$ = 12.3 Hz, $J_{1',1}$ = 4.5 Hz, C$\underline{H}$-1'), 4.08 (dd, 1H, $J_{1'',1'}$ = 12.3 Hz, $J_{1'',1}$ = 2.4 Hz, C$\underline{H}$-1"), 3.96-3.90 (m, 1H, C$\underline{H}$-5), 3.77 and 3.76 (2s, 6H, 2xOC$\underline{H}_3$), 2.38 (t, 2H, $J_{4',5'}$ = 7.5 Hz, C$\underline{H}_2$-4'), 2.06, 1.99, 1.97 and 1.91 (4s, 12H, 4xC=OC$\underline{H}_3$), 1.46-1.33 (m, 2H, C$\underline{H}_2$-5'), 1.22 (br s, 22H, (C$\underline{H}_2$)$_{11}$), 0.84 (t, 3H, J = 6.6 Hz, C$\underline{H}_3$-chain); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 171.2, 170.6, 170.0, 169.3, 168.7 and 167.1 (6x$\underline{C}$=O), 144.8 and 130.4 ($\underline{C}$=$\underline{C}$), 97.5 ($\underline{C}$-1), 71.2 ($\underline{C}$-4), 68.4 ($\underline{C}$-5), 68.0 ($\underline{C}$-3), 63.7 ($\underline{C}$-6), 61.9 ($\underline{C}$-1'), 52.5 and 52.4 (2xO$\underline{C}H_3$), 51.7 ($\underline{C}$-2) 31.9, 30.5, 29.7, 29.49, 29.45, 29.4, 28.4 and 22.7 (13x$\underline{C}H_2$-chain), 23.1 ($NHC{=}O\underline{C}H_3$), 20.7 and 20.6 ($3xC{=}O\underline{C}H_3$), 14.1 ($\underline{C}H_3$-chain); HRMS (ES+) Calcd for $C_{35}H_{57}NO_{13}Na$ 722.3728, found 722.3729 $[M+Na]^+$.

3,4,6-Tri-O-acetyl-2-azido-2-deoxy-D-glucopyranoside (93).



The known compound **93**[175,413,415,426,427] was prepared based on a deacetylation method described by Excoffier.[215] To a solution of **54** (881 mg, 2.35 mmol) in DMF (1 mL) at 50 °C was added hydrazine acetate (260 mg, 2.83 mmol). The mixture was stirred at 50 °C for 4 min and at rt for 10 min. The reaction mixture was then diluted with EtOAc (60 mL), washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure to give the desired product **93** (771 mg, 99%) as a colorless foam: IR (neat) (lit.[426]) 3400, 2100, 1750 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) (α-D:β-D/3:2) δ 5.51 (dd, 0.6H, $J_{3,2} = 10.5$ Hz, $J_{3,4} = 9.3$ Hz, C$\underline{H}$-3), 5.39 (d, 0.6H, $J_{1,2} = 3.6$ Hz, C$\underline{H}$-1), 5.07-4.97 (m, 1.4H, C$\underline{H}$-2 and 2xC$\underline{H}$-4), 4.73 (d, 0.4H, $J_{1,2} = 8.1$ Hz, C$\underline{H}$-1), 4.30-4.05 (m, 3H, C$\underline{H}$-5, C$\underline{H}$-6, and C$\underline{H}$-6'), 3.47 (dd, 0.4H, $J_{2,3} = 10.2$ Hz, $J_{2,1} = 8.1$ Hz, C$\underline{H}$-2), 3.43 (dd, 0.6H, $J_{2,3} = 10.5$ Hz, $J_{2,1} = 3.6$ Hz, C$\underline{H}$-2), 2.08, 2.07, 2.03 and 2.01 (4s, 9H, $3xC{=}OC\underline{H}_3$).

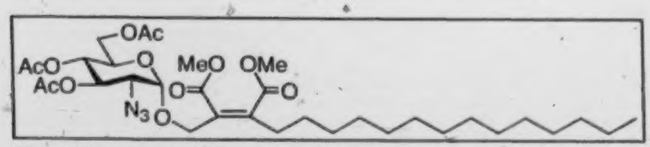
O-(Dimethyl (Z)-2-oxymethyl-3-octylbutenedioate) 3,4,6-tri-O-acetyl-2-azido-2-deoxy-α-D-glucopyranoside (94).



A mixture of α-imidate **85** (110 mg, 0.38 mmol) and alcohol **73** (202 mg, 0.42 mg) in dry Et_2O

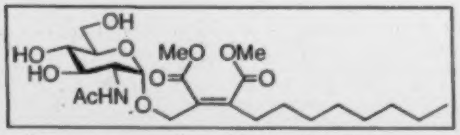
(13 mL) was stirred in the presence of powdered activated 4 Å molecular sieves for 30 min. TMSOTf (34 mg, 27 μL, 0.16 mmol) was added to the reaction mixture at -20 °C. After being stirred at -20 °C for 3.5 h, the reaction mixture was diluted with EtOAc, and filtered through Celite®. The filtrate was washed with a saturated aq. $NaHCO_3$ solution, and brine, dried (Na_2SO_4), and concentrated under reduced pressure. Further purification by preparative TLC (SiO_2, 1:1/hexane:Et_2O) gave **94** (161 mg, 70%) as a colorless oil: $[\alpha]_D^{26}$ +85° (*c* 0.22, $CHCl_3$); IR ($CHCl_3$ cast) 3020, 2927, 2854, 2111, 1735, 1216, 1047, 758, 669 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.41 (dd, 1H, $J_{3,2}$ = 10.8 Hz, $J_{3,4}$ = 9.3 Hz, C$\underline{H}$-3), 5.04 (dd, 1H, $J_{4,5}$ = 9.9 Hz, $J_{4,3}$ = 9.3 Hz, C$\underline{H}$-4), 5.03 (d, 1H, $J_{1,2}$ = 3.6 Hz, C$\underline{H}$-1), 4.55 (d, 1H, $J_{6,6'}$ = 11.4 Hz, C$\underline{H}$-6), 4.37 (d, 1H, $J_{6',6}$ = 11.4 Hz, C$\underline{H}$-6'), 4.28 (dd, 1H, $J_{1',1''}$ = 12.6 Hz, $J_{1',1}$ = 4.5 Hz, C$\underline{H}$-1'), 4.08 (dd, 1H, $J_{1'',1'}$ = 12.6 Hz, $J_{1'',1}$ = 2.4 Hz, C$\underline{H}$-1"), 4.03 (ddd, 1H, $J_{5,4}$ = 9.9 Hz, $J_{5,6}$ = 4.2 Hz, $J_{5,6'}$ = 2.1 Hz, C$\underline{H}$-5), 3.79 and 3.77 (2s, 6H, 2xOC$\underline{H}_3$), 3.32 (dd, 1H, $J_{2,3}$ = 10.8 Hz, $J_{2,1}$ = 3.6 Hz, C$\underline{H}$-2), 2.45 (t, 2H, $J_{4',5'}$ = 7.5 Hz, C$\underline{H}_2$-4'), 2.08, 2.07 and 2.03 (3s, 9H, 3xC=OC$\underline{H}_3$), 1.50-1.40 (m, 2H, C$\underline{H}_2$-5'), 1.25 (br s, 10H, (C$\underline{H}_2$)$_5$), 0.87 (t, 3H, J = 6.9 Hz, C$\underline{H}_3$-chain); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 170.6, 169.9, 169.7, 169.1 and 166.7 (5x$\underline{C}$=O), 147.1 and 128.9 ($\underline{C}$=$\underline{C}$), 97.5 ($\underline{C}$-1), 70.3 ($\underline{C}$-3), 68.5 ($\underline{C}$-4), 68.1 ($\underline{C}$-5), 63.3 ($\underline{C}$-6), 61.8 ($\underline{C}$-1'), 60.8 ($\underline{C}$-2), 52.5 and 52.4 (2xO$\underline{C}H_3$), 31.8, 30.9, 29.7, 29.5, 29.2, 28.1 and 22.7 (7x$\underline{C}H_2$-chain), 20.74, 20.71 and 20.6 (3xC=O$\underline{C}H_3$), 14.1 ($\underline{C}H_3$-chain); HRMS (ES+) Calcd for $C_{27}H_{41}N_3O_{12}Na$ 622.2588, found 622.2579 $[M+Na]^+$.

O-*(Dimethyl (Z)-2-oxymethyl-3-tetradecylbutenedioate) 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-*D*-glucopyranoside* (95).



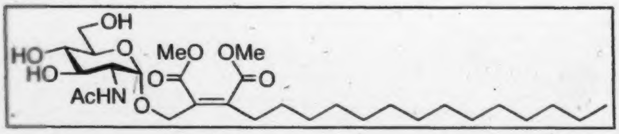
The reaction of the α-imidate **85** (55 mg, 0.12 mmol), the alcohol **74** (33 mg, 0.09 mmol), and TMSOTf (8 mg, 6 μL, 0.04 mmol) was performed as described for the synthesis of **94**. Further purification by flash chromatography (SiO_2; 6:2.5/hexane:EtOAc, R_f 0.32 (5:3/hexane:EtOAc)) gave **95** (42 mg, 70%) as a colorless oil: $[\alpha]_D^{26}$ +69° (*c* 1.44, $CHCl_3$); IR ($CHCl_3$ cast) 2925, 2854, 2110, 1751, 1643, 1435, 1367, 1227, 1047 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 5.39 (dd, 1H, $J_{3,2}$ = 10.5 Hz, $J_{3,4}$ = 9.3 Hz, C$\underline{H}$-3), 5.02 (dd, 1H, $J_{4,5}$ = 10.2 Hz, $J_{4,3}$ = 9.3 Hz, C$\underline{H}$-4), 5.01 (d, 1H, $J_{1,2}$ = 3.6 Hz, C$\underline{H}$-1), 4.53 (d, 1H, $J_{6,6'}$ = 11.7 Hz, C$\underline{H}$-6), 4.35 (d, 1H, $J_{6',6}$ = 11.7 Hz, C$\underline{H}$-6'), 4.27 (dd, 1H, $J_{1',1''}$ = 12.3 Hz, $J_{1',1}$ = 4.5 Hz, C$\underline{H}$-1'), 4.06 (dd, 1H, $J_{1'',1'}$ = 12.3 Hz, $J_{1'',1}$ = 2.4 Hz, C$\underline{H}$-1"), 4.01 (ddd, 1H, $J_{5,4}$ = 10.2 Hz, $J_{5,6}$ = 4.2 Hz, $J_{5,6'}$ = 2.1 Hz, C$\underline{H}$-5), 3.77 and 3.75 (2s, 6H, 2xOC$\underline{H}_3$), 3.30 (dd, 1H, $J_{2,3}$ = 10.5 Hz, $J_{2,1}$ = 3.6 Hz, C$\underline{H}$-2), 2.44 (t, 2H, $J_{4',5'}$ = 7.8 Hz, C$\underline{H}_2$-4'), 2.06, 2.05 and 2.01 (3s, 9H, 3xC=OC$\underline{H}_3$), 1.48-1.37 (m, 2H, C$\underline{H}_2$-5'), 1.23 (br s, 22H, (C$\underline{H}_2)_{11}$), 0.86 (t, 3H, J = 6.6 Hz, C$\underline{H}_3$-chain); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 170.7, 170.1, 169.9, 169.3 and 166.8 (5x$\underline{C}$=O), 147.3 and 129.1 ($\underline{C}$=$\underline{C}$), 97.6 ($\underline{C}$-1), 70.5 ($\underline{C}$-3), 68.6 ($\underline{C}$-4), 68.2 ($\underline{C}$-5), 63.4 ($\underline{C}$-6), 61.9 ($\underline{C}$-1'), 60.9 ($\underline{C}$-2), 52.6 and 52.5 (2xO$\underline{C}H_3$), 32.1, 31.1, 30.7, 29.8, 29.7, 29.63, 29.55, 29.48, 28.5, 28.3 and 22.9 (13x$\underline{C}H_2$-chain), 20.88, 20.85 and 20.8 (3xC=O$\underline{C}H_3$), 14.3 ($\underline{C}H_3$-chain); HRMS (ES+) Calcd for $C_{33}H_{57}N_4O_{12}$ 701.3973, found 701.3973 $[M+NH_4]^+$.

O-*(Dimethyl (Z)-2-oxymethyl-3-octylbutenedioate) 2-acetamido-2-deoxy-α-D-glucopyranoside* (96).



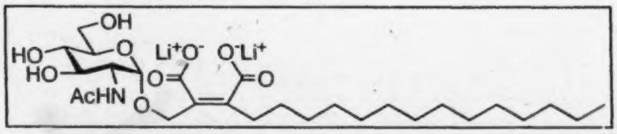
To **90** (15 mg, 0.02 mmol) in dry MeOH (1 mL) was added sodium (0.2 mg). The reaction mixture was stirred at rt for 1 h, neutralized by addition of Amberlite IR-120 (H^+) resin, filtered, and concentrated under reduced pressure. Further purification by reversed-phase HPLC (C_{18} Bondpak, flow rate: 15 mL/min, gradient elution: 40-80% MeCN in H_2O over 9 min, 80-100% MeCN in H_2O over 1 min) gave **96** (t_R 6.2 min, 12 mg, 99%) as a colorless oil: ^{1}H NMR (CD_3OD, 300 MHz) δ 7.89 (d, 1H, $J_{NH,2}$ = 8.7 Hz, N$\underline{H}$), 4.81 (s 1H, C$\underline{H}$-1), 4.49 (d, 1H, $J_{6,6'}$ = 11.7 Hz, C$\underline{H}$-6), 4.24 (d, 1H, $J_{6',6}$ = 11.7 Hz, C$\underline{H}$-6'), 3.84 (dd, 1H, $J_{2,3}$ = 10.8 Hz, $J_{2,1}$ = 3.6 Hz, C$\underline{H}$-2), 3.78 (d, 1H, $J_{1',1}$ = 2.1 Hz, C$\underline{H}$-1'), 3.72 (2s, 6H, 2xOC$\underline{H}_3$), 3.68 (dd, 1H, $J_{1'',1'}$ = 12.0 Hz, $J_{1'',1}$ = 5.4 Hz, C$\underline{H}$-1"), 3.57 (dd, 1H, $J_{3,2}$ = 10.8 Hz, $J_{3,4}$ = 8.7 Hz, C$\underline{H}$-3), 3.54 (ddd, 1H, $J_{5,4}$ = 9.6 Hz, $J_{5,6}$ = 5.1 Hz, $J_{5,6'}$ = 2.1 Hz, C$\underline{H}$-5), 3.34 (dd, 1H, $J_{4,5}$ = 9.6 Hz, $J_{4,3}$ = 8.7 Hz, C$\underline{H}$-4), 2.44 (t, 2H, $J_{4',5'}$ = 7.2 Hz, C$\underline{H}_2$-4'), 1.94 (s, 3H, C=OC$\underline{H}_3$), 1.45-1.33 (m, 2H, C$\underline{H}_2$-5'), 1.27 (br s, 10H, (C$\underline{H}_2$)$_5$), 0.87 (t, 3H, J = 6.3 Hz, C$\underline{H}_3$-chain); HRMS (ES+) Calcd for $C_{23}H_{39}NO_{10}Na$ 512.2472, found 512.2474 $[M+Na]^+$.

O-*(Dimethyl (Z)-2-oxymethyl-3-tetradecylbutenedioate) 2-acetamido-2-deoxy-α-*D-*glucopyranoside* (97).



A solution of **91** (35 mg, 49 μmol) in MeOH (1 mL) was stirred at rt in the presence of a catalytic amount of NaOMe for 1 h. The reaction mixture was then neutralized with Amberlite IR-120 (H^+) resin, filtered, and concentrated under reduced pressure. Further purification by reversed-phase HPLC (C_{18} Bondpak, flow rate: 15 mL/min, gradient elution: 20% MeCN in H_2O for 2 min, 20-80% MeCN in H_2O over 10 min, 100% MeCN for 5 min, 100-20% MeCN in H_2O over 2 min) gave **97** (t_R 13.7 min, 28 mg, 99%) as a white solid: ^1H NMR ($CDCl_3$, 300 MHz) δ 6.38 (d, 1H, $J_{NH,2}$ = 7.8 Hz, N$\underline{H}$), 4.81 (d, 1H, $J_{1,2}$ = 3.9 Hz, C$\underline{H}$-1), 4.47 (d, 1H, $J_{6,6'}$ = 12.3 Hz, C$\underline{H}$-6), 4.27 (d, 1H, $J_{6',6}$ = 12.3 Hz, C$\underline{H}$-6'), 4.03-3.96, 3.86-3.80 and 3.69-3.55 (3m, 5H, O$\underline{H}$, C$\underline{H}$-2, C$\underline{H}$-3, C$\underline{H}$-4 and C$\underline{H}$-5), 3.78 and 3.77 (2s, 6H, 2xOC$\underline{H}_3$), 3.65 (d, 1H, $J_{1',1''}$ = 4.5 Hz, C$\underline{H}$-1'), 3.59 (d, 1H, $J_{1'',1'}$ = 4.5 Hz, C$\underline{H}$-1"), 3.52 (br s, 1H, O$\underline{H}$), 2.69 (br s, 1H, O$\underline{H}$), 2.38 (t, 2H, $J_{4',5'}$ = 7.5 Hz, C$\underline{H}_2$-4'), 2.05 (s, 3H, C=OC$\underline{H}_3$), 1.46-1.32 (m, 2H, C$\underline{H}_2$-5'), 1.23 (br s, 22H, (C$\underline{H}_2$)$_{11}$), 0.86 (t, 3H, J = 6.9 Hz, C$\underline{H}_3$-chain); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 172.7, 168.6 and 167.9 (3x$\underline{C}$=O), 143.6 and 131.5 ($\underline{C}$=$\underline{C}$), 97.6 ($\underline{C}$-1), 73.9, 72.0 and 71.4 ($\underline{C}$-3, $\underline{C}$-4 and $\underline{C}$-5), 63.4 ($\underline{C}$-6), 62.0 ($\underline{C}$-1'), 52.5 and 52.4 (2xO$\underline{C}H_3$), 54.0 ($\underline{C}$-2), 32.0, 30.2, 29.70, 29.65, 29.54, 29.46, 29.40, 28.6 and 22.7 (13x$\underline{C}H_2$-chain), 23.1 (C=O$\underline{C}H_3$), 14.1 ($\underline{C}H_3$-chain); HRMS (ES+) Calcd for $C_{29}H_{51}NO_{10}Na$ 596.3411, found 596.3419 $[M+Na]^+$.

***(Z)-2-(2-Acetamido-2-deoxy-α-D-glucopyranosyl)oxymethyl-3-tetradecylbutenedioic acid dilithium salt* (98).**



1 N LiOH (38 μL, 2.3 eq) was added to compound **97** (9.5 mg, 17 μmol) in a THF:H_2O/1:1 solution (1 mL). The reaction mixture was stirred at rt for 21 h. The solvent was removed under reduced pressure and the remaining solid was dissolved in H_2O. Non-polar impurities including unreacted starting material were removed by extraction with CH_2Cl_2. Freeze-drying of the aqueous layer followed by reversed-phase HPLC (C_{18} Bondpak, flow rate: 15 mL/min, gradient elution: 30% MeCN in H_2O) gave **98** (t_R 6.6 min, 8.5 mg, 90%) as a white solid. HRMS (ES+) Calcd for $C_{27}H_{47}NO_{10}Li$ 552.3360, found 552.3357 $[MH_2+Li]^+$.

RUBBER TRANSFERASE INHIBITION STUDIES WITH CHAETOMELLIC ACID A ANALOGS 13-16

1. Reagents for Rubber Transferase Assays

Farnesyl diphosphate (FPP), dimethyl allyl diphosphate (DMAPP) and [1-^{14}C]-IPP (55mCi/mmol) were purchased from American Radiolabeled Chemicals Inc. (St. Louis, Missouri, USA). Washed rubber particles (WRP) from *P. argentatum* and *H. brasiliensis* were purified[428,429] and stored in liquid nitrogen.[430]

2. Rubber Transferase Assays

Rubber transferase assays were all performed in triplicate by Christopher J. D. Mau (USDA, Agricultural Research Service, Western Regional Research Center, Albany, CA) as previously described.[148] Concentrated stock solutions (2 mM) of chaetomellic acid A analogs **13-16** were prepared in DMSO. The typical reaction assay (50 μL) contained [1-^{14}C]-IPP (1 mM) (0.19 mCi/mmol), $MgSO_4$ (1.25 mM), DTT (5 mM), and Tris pH 7.5 (100 mM). Various concentrations of initiators (FPP or DMAPP) were added, ranging from 10 pM to 1 mM, along with the stated concentration of derivatives **13-16**. The reactions were incubated for 4 h at 16 °C for *P. argentatum* WRP (0.25 mg) and at 25 °C for *H. brasiliensis* WRP (0.5 mg), and were stopped by addition of EDTA (0.5 M pH 8) to a final concentration of 20 mM. The incorporated ^{14}C was measure by liquid scintillation counting of the newly synthesized rubber that had been trapped on filters and subsequently washed to remove unincorporated ^{14}C-IPP. Because analogs **13-16** were prepared as lithium salts, assays were compared to internal control containing comparable amounts of LiCl and DMSO.

GENERAL METHODS FOR GENETIC MANIPULATIONS AND BIOLOGICAL EXPERIMENTS

1. Bacterial strains, plasmids, culture media, and growth conditions

The plasmids and producer strains utilized in this study are listed in Table 9. Bacteria were maintained as frozen stock cultures at -70 °C in Bacto APT broth (All Purpose Tween, Difco Laboratories Inc., Detroit, Mich.) supplemented with 20% (vol/vol) glycerol, except strains of *E. coli*, which were stored in LB broth (Luria-Bertani) under the same conditions. Prior to experimental use, *E. coli* strains were subcultured twice in LB broth and grown overnight with shaking at 37 °C. All other strains were subcultured twice and grown overnight at 25 °C in APT broth (first day), and APT broth (*Carnobacterium piscicola* 307 and 682) or modified semidefined Casamino Acids medium (CAA)[329] (brochocin producer) (second day). Celtone®-U complete medium (5013L0) was purchased from Spectra Stable Isotopes (Columbia, MD, USA) and used as supplied. Transformants of *E. coli* were selected on LB agar (Difco) with a selective concentration of ampicillin (150 µg/mL). For growth of *Carnobacterium* transformants on APT agar, erythromycin (5 µg/mL) was used. Solid and soft agar media were prepared by addition of granulated agar 1.5% and 0.75% (wt/vol), respectively, to the broth media.

2. Reagents and solutions for DNA isolation and manipulation

All genetic manipulations and biological experiments were performed under sterile conditions (material and solutions autoclaved at 121 °C for 10-15 min). Restriction enzymes *Xba*I and *Eco*RI were purchased from Gibco BRL (Burlington, Ontario, Canada), *Stu*I and *Xmn*I from New England Biolabs Inc. (Mississauga, Ontario, Canada), and *Sal*I from Invitrogen (Burlington, Ontario, Canada). Restriction enzyme digestions were performed according to the manufacturer's instructions. T4 DNA ligase was purchased from Invitrogen, New England Biolabs Inc. or Promega (Burlington, Ontario, Canada)]. Ligations and *E. coli* transformations were done using standard methods.[431] Oligonucleotides prepared as sequencing and PCR primers were synthesized on an Applied Biosystems (model 391) PCR Mate synthesizer (Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada). Agarose gel (1.8% in TBE containing 5 µl EtBr/100 mL) electrophoresis was conducted with Tris-borate-EDTA (TBE) buffer (Sigma) at 110 V. The construction of MBP fusion protein of BrcA in *E. coli* was done using the Protein Fusion & Purification (pMAL™) System #E8000S (New England Biolabs Inc.). The construction of intein fusion protein of BrcB in *E. coli* was achieved using the IMPACT™-CN System #6900S (New England Biolabs Inc.).

3. General procedure for isolation of DNA from *E. coli* on large scale[276,431]

A culture of the desired clone in *E. coli* was grown in sterile LB broth [ampicillin (150 µg/mL)] (200 mL) with shaking at 37 °C. After 20 h of incubation, cells were isolated from the culture broth by centrifugation (8000 rpm, 10 min, 4 °C) and resuspended in sterile STE buffer (NaCl:Tris pH 8.0:EDTA/100:10:1 mM, 20 mL). After centrifugation (8000 rpm, 10 min, 4 °C) the cells were resuspended in solution A (glucose:Tris-HCl pH 8.0:EDTA/50:25:10 mM, 7.5 mL). Digestion of the polymeric compounds found in the cell wall was performed by addition of lysozyme (4 mg/mL, 32 mg) to the mixture at 37 °C for 45 min. Freshly prepared solution B (0.2 N NaOH:1%

SDS, 16 mL) was then added and the mixture was swirled gently until it became clear. Protein and chromosomal DNA precipitation was performed by addition of cold (4 °C) solution C (3 M KOAc:glacial AcOH:H_2O/60:11.5:28.5 mL, 12 mL) to the mixture. After centrifugation (8000 rpm, 10 min, 4 °C), the supernatant was transferred into two 30 mL sterile centrifugation tubes and centrifuged again (12000 rpm, 10 min, 4 °C) to remove any remaining white particles. The supernatant was transferred into two clean 30 mL sterile centrifugation tubes each containing 2-propanol (15 mL) to allow DNA precipitation at rt for 15 min. Crude DNA was isolated by centrifugation (12000 rpm, 15 min, 15 °C). The crude DNA pellets were washed really gently with cold (4 °C) 70% EtOH (2x500 μL), dried under vacuum (med rate, 5-10 min), and dissolved in sterile TE buffer (Tris:EDTA/10:1 mM, 3 mL in each tube, 10 min at rt). The content of both tubes was combined, CsCl (1 g/mL, 6 g) was added, and the solution was added to a tube containing EtBr (300 μL). Pure DNA was isolated by EtBr/CsCl density gradient centrifugation[431] (49000 rpm, 20 h, 20 °C) followed by isoamyl alcohol wash (5x1.5 mL), dialysis in TE buffer (3-4 h, 500 mL replaced every hour), phenol extraction (1:1/DNA solution:phenol), $CHCl_3$:isoamyl alcohol/24:1 extraction (1:1/DNA solution:$CHCl_3$ solution), and DNA precipitation (1:2/DNA solution:EtOH 96% + NaOAc 3 M pH 5.0, 2 h, 4 °C). Pure DNA was finally obtained by centrifugation (12000 rpm, 30 min, 4 °C). The pure DNA pellets were washed really gently with cold (4 °C) 70% EtOH (2x500 μL), dried under vacuum (med rate, rotor on, 5-10 min), and dissolved in sterile TE buffer (100 μL total) for 10 min at rt and kept at -20 °C.

4. General procedure for isolation of DNA from lactic acid bacteria (LAB) on large scale

A culture of the desired clone in LAB was grown in sterile APT broth [erythromycin (5 μg/mL)] (400 mL) without shaking at 25 °C. After 24 h of incubation, cells were isolated from the culture broth by centrifugation (8000 rpm, 10 min, 4 °C) and resuspended in sterile STE buffer (NaCl:Tris pH 8.0:EDTA/100:10:1 mM, 20 mL). After centrifugation (8000 rpm, 10 min, 4 °C) the cells were resuspended in solution A (sucrose:Tris-HCl pH 8.1:EDTA:NaCl/20%:10:10:50 mM, 7.5 mL). Digestion of the polymeric compounds found in the cell wall was performed by addition of lysozyme (15 mg/mL, 120 mg) to the mixture at 37 °C for 1 h. Freshly prepared solution B (0.2 N NaOH:1% SDS, 16 mL) was then added and the mixture was swirled gently until it became clear. Protein and chromosomal DNA precipitation was performed by addition of cold (4 °C) solution C (5 M KOAc:glacial AcOH:H_2O/60:11.5:28.5 mL, 12 mL) to the mixture. After centrifugation (8000 rpm, 10 min, 4 °C), the supernatant was transferred into two 30 mL sterile centrifugation tubes and centrifuged again (12000 rpm, 10 min, 4 °C) to remove any remaining white particles. A mixture of phenol:($CHCl_3$:isoamyl alcohol/24:1)/1:5 mL was then added to the supernatant. The resulting solution was swirled gently and the emulsion was separated by centrifugation (12000 rpm, 15 min, rt). The upper layer was transferred into two clean 30 mL sterile centrifugation tubes each containing 2-propanol (15 mL) to allow DNA precipitation at rt for 10 min. Crude DNA was isolated by centrifugation (12000 rpm, 15 min, 15 °C). The crude DNA pellets were washed really gently with cold (4 °C) 70% EtOH (10 mL/tube), dried under vacuum (med rate, 5-10 min), and dissolved in sterile TE buffer (Tris:EDTA/10:1 mM, 3 mL in each tube, 10 min at rt). From this point, EtBr/CsCl and isolation of the pure DNA was performed as described for DNA isolation on large scale from *E. coli* (Section 3 of the General Methods).

5. General procedure for preparation of competent *E. coli* cells

A culture of the desired *E. coli* cells (1% inoculum) was grown in sterile LB broth [ampicillin (150 μg/mL)] (100 mL) with shaking at 37 °C. After 2 h of incubation, the culture was separated into 2 mL samples and cells were isolated from the culture broth by centrifugation (12000 rpm, 1 min, rt). The pellets were resuspended (vortex for 5 sec) in iced-cold 0.1 M $CaCl_2$ (1 mL) and kept on ice for 30-45 min. The cells were isolated by centrifugation (12000 rpm, 30 sec, 4 °C), resuspended (vortex for 3 sec) in an iced-cold solution of 0.1 M $CaCl_2$:10% glycerol (0.2 mL), and kept at -70 °C.

6. General procedure for transformation of *E. coli* cells

After complete ligation, the ligation mixture was kept on ice. The desired competent *E. coli* cells, kept at -70 °C, were thawed on ice (~5 min). All the ligation mixture was then added to the thawed competent cells, and the mixture was kept on ice for another 30 min. After a heat-shock treatment at 37 °C for 5 min (precise), LB broth (500 μL) was added and the resulting mixture was kept at 37 °C for 1 h (precise). The *E. coli* transformants (100 μL/plate) were then grown at 37 °C for 20 h on LB agar with a selective concentration of ampicillin (150 μg/mL).

7. General procedure for isolation of crude DNA as template for PCR from *E. coli* or *C. piscicola* on mini-preparative scale

A culture of the desired *E. coli* cells was grown in sterile LB broth [ampicillin (150 μg/mL)] (5 mL) with shaking at 37 °C. For the growth of *C. piscicola* cells, APT broth (5 mL) was used at 25 °C without shaking. After 20-24 h of incubation, a 1 mL sample was taken and cells were isolated from the culture broth by centrifugation (12000 rpm, 5 min, rt). The pellets were resuspended (vortex for 5 sec) in H_2O (1 mL) and the cells were isolated by centrifugation (12000 rpm, 5 min, rt). The cells were resuspended (vortex for 5 sec) in H_2O (100 μL), boiled for 15 min, and kept at -70 °C as concentrated crude DNA template for PCR experiments.

8. Screening for the desired clones after transformations of *E. coli* cells

Two methods were used for screening for the desired clones after transformation of *E. coli* cells.

Method A**:** *Step 1. DNA Isolation*: Colonies were grown in LB broth [ampicillin (150 μg/mL)] (2.5 mL) with shaking at 37 °C. After 20-24 h of incubation, 1.5 mL samples were taken and cells were isolated from the culture broth by centrifugation (12000 rpm, 30 sec, rt). The pellets were resuspended (vortex for 30 sec) in H_2O (200 μL) and the cells were isolated by centrifugation (12000 rpm, 30 sec, rt). The pellets were resuspended (vortex for 10 sec) in freshly prepared solution A (Tris-HCl pH 8.0:EDTA/50:10 mM, 200 μL). Freshly prepared solution B (0.2 N NaOH:1% SDS, 200 μL) was then added and the mixture was swirl gently until it became clear. Protein and chromosomal DNA precipitation was performed by addition of cold (4 °C) solution C (5

M KOAc:glacial AcOH:H_2O/60:11.5:28.5 mL, 200 μL) to the mixture. After centrifugation (12000 rpm, 10 min, 4 °C), the supernatant was transferred into a new 1.5 mL eppendorf. Phenol:($CHCl_3$:isoamyl alcohol/24:1)/200:200 μL extraction, $CHCl_3$:isoamyl alcohol/24:1 (400 μL) extraction, and DNA precipitation (EtOH 96% (800 μL), at least 10 min, -20 °C) were then performed. Pure DNA was finally obtained by centrifugation (12000 rpm, 30 min, 4 °C). The pure DNA pellets were washed really gently with cold (4 °C) 70% EtOH (2x500 μL), dried under vacuum (med rate, rotor on, 5-6 min), and dissolved in sterile TE buffer [RNAse (20 μg/mL)] (25 μL total) for 10 min at rt and kept at -20 °C.

Step 2. Screening: Digestion using the endonucleases that were utilized for the cloning experiments allowed to see, by agarose gel (1.8% in TBE containing 5 ml EtBr/100 mL), which constructs contained the desired clone.

Method B: *Step 1. DNA Isolation*: Colonies were grown in LB broth [ampicillin (150 μg/mL)] (2.5 mL) with shaking at 37 °C. Their DNA was then isolated on mini-preparative scale (Section 7 of the General Methods).

Step 2. DNA Amplification: DNA amplification by PCR experiments were performed (Section 9 of the General Methods) using primer that allowed for amplification of the inserted gene in the plasmid. Agarose gel (1.8% in TBE containing 5 mL EtBr/100 mL) on the PCR products allowed for detection of constructs containing the desired gene.

9. Polymerase chain reaction (PCR)

DNA amplification by PCR experiments were performed on a Perkin Elmer GeneAmp PCR System 2400 using 100 μL samples containing (per 100 μL): 10X High

Fidelity PCR Buffer (600 mM Tris-SO_4 (pH 8.9), 180 mM $(NH_4)_2SO_4$), 10 µL; 10 mM dNTP, 2 µL; 50 mM $MgSO_4$, 4 µL; primers, 1 µL each; template DNA, 1 µL; autoclaved distilled MQ H_2O, 80 µL; Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen), 0.5 µL added after a thermal cycle at 94 °C for 2 min to completely denaturate the template and activate the enzyme. Taqplus precision polymerase and 50 mM $MgCl_2$ (Stratagene, Aurora, Ontario, Canada), where specified, were used to perform DNA amplification. In general 25-30 cycles of PCR amplifications were performed and the reaction mixture was maintained at 4 °C after the final cycle of 5 min at 72 °C.

Purification of DNA from PCR reactions was done by dilution to 230 µL of the reaction mixture with sterile MQ H_2O, phenol extraction (200 µL), $CHCl_3$:isoamyl alcohol/24:1 extraction (200 µL), and DNA precipitation (EtOH 96%: NaOAc 3 M pH 5.0/400:25 µL, 12-16 h, 4 °C). Isolation of pure DNA was accomplished by centrifugation (12000 rpm, 30 min, 4 °C) followed by gentle wash with cold (4 °C) 70% EtOH (2x500 µL) and drying under vacuum (med rate, rotor on, 5-10 min) of the pure DNA pellets which were then dissolved in sterile TE buffer (20 µL) for 10 min at rt and kept at -20 °C. Where specified, purification of DNA from PCR experiments was performed using a QIAquick™ PCR Purification Kit #28104 (QIAGEN, Mississauga, Ontario, Canada).

10. Mass spectrometry

Mass spectrometry analyses were performed with a linear matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometer (Applied Biosystems Voyager Elite). All spectra were recorded in positive ion mode with an acceleration voltage of 20 kV in the presence of a nitrogen laser (λ = 337 nm) used for desorption/ionization of the samples. Samples were prepared using α-cyano-4-hydroxycinnamic acid (Aldrich) or sinapinic acid (Aldrich) as a matrix, and fixed to a gold target before analysis. Bovine insulin (MH^+ = 1046.542, Sigma) was used for calibration of the instrument, which was performed before each experiment.

11. N-Terminal amino acid sequencing

The amino acid sequences of bacteriocins was established by the Alberta Peptide Institute (University of Alberta, Edmonton, Alberta, Canada) by automated Edman degradation with a gas-phase protein sequencer (Applied Biosystems model 470A) with an on-line model 120A phenylthiohydantoin amino acid analyzer.

12. Circular dichroism

Circular dichroism measurements on purified samples of BrcA and BrcB(10-43) in methanol were performed (cell length 0.02 cm) in the absence and the presence of 50% TFE between 250-188 nm at 25 °C using a JACSO J720 spectropolarimeter equipped with JASCO J700 software (done by R. Luty, Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada).

13. Nucleotide sequence accession numbers

The nucleotide sequence reported for brochocin C has been assigned GenBank™ (Los Almos, NM) accession number AF075600.[277] The nucleotide sequence reported for carnobacteriocin BM1 has been assigned GenBank™ (Los Almos, NM) accession number L29058.[306] The nucleotide sequence reported for piscicolin 126 has been assigned SWISS-PROT database accession number P80569.[307]

GENETIC MANIPULATIONS AND BIOLOGICAL EXPERIMENTS

1. Isolation of various bacteriocins including piscicolin 126 and carnobacteriocins B1 and BM1 (CbnB1 and CbnBM1) from *C. piscicola* 307 and 682

Cultures of *C. piscicola* 307 and 682 (1% inoculum) were grown without shaking in sterile APT (1 L) at 25 °C. After 21-24 h of incubation, cells were removed from the culture broth by centrifugation (8000 rpm, 20 min, 4 °C). At 4 °C, the supernatant was passed through an Amberlite XAD-16 resin column (2.5 x 50 cm, 60 g of resin) at a flow rate of 15 mL/min using a peristaltic pump. The column was then washed with 30% EtOH (750 mL), and the active peptides were eluted using 70% IPA (1 L, adjusted to pH 2 with 1 M HCl). The IPA was evaporated under reduced pressure at low temperature (< 30 °C). The residue was suspended in H_2O (25 mL) and loaded onto a Varian MEGA BE-C_{18} column (10 g, 60 mL, #12256031) pre-washed with MeOH and H_2O (1 column volume each). The column was successively washed at a flow rate of ~5 mL/min with H_2O, 30% EtOH, 20% IPA (1 column volume each), and 40% IPA (0.5 column volume). The active peptides were eluted using 70% IPA (100 mL, adjusted to pH 2 with 1 M HCl). The IPA was evaporated under reduced pressure at low temperature (< 30 °C). The residue was suspended in H_2O (2 mL) and loaded at 4 °C onto a Sephadex G25 superfine (Amersham Pharmacia Biotech, Québec, Canada) column (3 x 45 cm) and eluted for 12 h at 0.4 mL/min (15 min/tube) with H_2O. All fractions collected were tested for antimicrobial activity by the spot-on-lawn assay, overlaying with *C. divergens* LV13.

Activity was found in fractions 25-29 (t_R 380-440 min). All active fractions were combined and the solvent was evaporated *in vacuo* at low temperature (< 30 °C). The residue was re-dissolved in 20% IPA (1.5 mL). Further purification by HPLC using a reversed-phase C_8 column (VYDAC™ 208TP54, 300 Å, 5 μm, 4.6 x 250 mm) on an analytical Beckman System Gold equipped with 32karat software was then required to isolate the various active peptides produced by each organisms. All fractions collected during HPLC experiments were kept on dry ice under argon to avoid loss of activity. They were all tested for antimicrobial activity against *C. divergens* LV13 and analyzed by mass spectrometry. Pure peptides were submitted for N-terminal amino acid sequencing. All peptides were detected at 220 nm, and eluted (150-400 μL injected) at 1 mL/min [X: H_2O (0.075% TFA), Y: IPA (0.075% TFA)] using the following protocols:

HPLC purification of peptides from C. piscicola 307: 4 peptides were isolated from *C. piscicola* 307: piscicolin 126, CbnB1, CbnBM1, and an unknown active bacteriocin. To achieve isolation of each peptide 3 HPLC experiments were required. A first elution (increase from 20 to 50% Y in 50 min; increase to 100% Y in 10 min; decrease to 20% Y in 2 min; stay at 20% Y for 5 min) gave 3 active fractions (t_R**A1** 10.3-12.7 min, t_R**B1** 12.7-16.2 min, and t_R**C1** 45.2-47.9 min). Fraction **C1** was pure and corresponded to the unknown active bacteriocin. Fraction **A1** was re-subjected to a second elution using the same gradient procedure. An active fraction (t_R**A2** 13.1-14.7 min) was found to contain CbnB1 and CbnBM1). CbnB1 was further purified and isolated in pure form. Fraction **B1** was also re-subjected to a second elution using the same gradient procedure. An active fraction (t_R**B2** 14.0-16.4 min) was found to contain CbnBM1 and piscicolin 126.

HPLC purification of peptides from C. piscicola 682: 2 peptides were isolated from *C. piscicola* 682: piscicolin 126 and CbnBM1. To achieve complete isolation of piscicolin 126, and partial isolation of CbnBM1, 5 HPLC experiments were required. A first elution (increase from 20 to 50% Y in 50 min; increase to 100% Y in 10 min; decrease to 20% Y in 2 min; stay at 20% Y for 5 min) gave 3 active fractions (t_R**D1** 14.0-16.3 min, t_R**E1** 16.3-18.1 min, and t_R**F1** 18.1-20.8 min). Fractions **D1**, **E1**, and **F1** were individually re-subjected to a second elution using the same gradient elution and active fractions (t_R**D2** 16.1-17.3 min, t_R**E2** 14.8-15.8 min, and t_R**F2** 15.8-18.1 min) were collected. Fraction **E2** was found to contain CbnBM1 and piscicolin 126. Fraction **E2** was re-injected onto the HPLC column and pure piscicolin 126 (t_R 15.5-16.9 min) was isolated.

2. Genetic characterization of *C. piscicola* 307 and 682

To confirm the presence of the genes encoding piscicolin 126 and CbnBM1 in the genetic sequence of *C. piscicola* 307 and 682, crude DNA from these two strains of carnobacteria was first isolated on mini-preparative scale (Section 7 of the General Methods). Primers LG-126 F1 (5'-ATATGAATTCCGATGTTACAATCAATTAAC-3') and LG-126F2 (5'-ATATGAATTCATGAAAACTGTTAAAGAACT-3') are complementary to the forward nucleotide sequence of piscicolin 126. Primers LG-126R1 (5'-ATATTCTAGACTTTTCCTCCAGAAAACCA-3') and LG-126R2 (5'-ATATTCTAGATTATCCTTTGTTCCAACC-3') are complementary to the reverse nucleotide sequence of piscicolin 126. DNA amplification by PCR experiments (denaturation, annealing, and extension temperatures (1 min each): 94, 50, and 68 °C, 30 cycles) of *C. piscicola* 682 using combination of primers LG-126F1 and LG-126R1, and

DNA amplification of *C. piscicola* 307 was performed using combinations of primers LG-126F1 and LG-126R1, as well as LG-126F2 and LG-126R1. Agarose gel electrophoresis and DNA sequencing using the combinations of primers utilized for PCR experiments confirmed the presence of the gene encoding piscicolin 126 in *C. piscicola* 307 and 682. Primer SG BM1-F1 (5'-ATATGAATTCATGAAAAGCGTTAAAGAACT-3') is complementary to the forward nucleotide sequence of CbnBM1. Primers SG BM1-R1 (5'-ATATTCTAGATTAATGTCCCATTCCTGC-3') and SG BM1-R2 (5'-ATATTCTAGATTAAAACCCTGACCAAGC-3') are complementary to the reverse nucleotide sequence of CbnBM1. DNA amplification by PCR experiments (denaturation, annealing, and extension temperatures (1 min each): 94, 52, and 68 °C, 25 cycles) of *C. piscicola* 307 and 682 was done using combination of primers SG BM1-F1 and SG BM1-R1, as well as SG BM1-F1 and SG BM1-R2. Purification of DNA from PCR experiments was in all cases performed using a QIAquick™ PCR Purification Kit #28104. Agarose gel electrophoresis and DNA sequencing using the combinations of primers utilized for PCR experiments could not confirm without ambiguity the presence of the gene encoding CbnBM1 in *C. piscicola* 307 and 682.

3. Genetic manipulations for verification of CbnB2 production by *C. piscicola* 307 and 682

To verify if the gene encoding CbnB2 is present in the genetic sequence of *C. piscicola* 307 and 682, crude DNA from these two strains of carnobacteria was first isolated on mini-preparative scale (Section 7 of the General Methods). Primer SG B2-F1 (5'-ATATTCTAGAATGAATAGCGTAAAAGAATTA-3') is complementary to the forward nucleotide sequence of CbnB2. Primers SG B2-R1 (5'-ATAATCTAGATTACGGTCTCCTACCAAT-3') and SG B2-R2 (5'-ATAATCTAGATTAGAAATATATATAAGGACCG-3') are complementary to the reverse nucleotide sequence of CbnB2. DNA amplification by PCR experiments (denaturation, annealing, and extension temperatures (1 min each): 94, 50, and 68 °C, 25 cycles) of *C. piscicola* 307 and 682 was performed using combinations of primers SG B2-F1 and SG B2-R1, as well as SG B2-F1 and SG B2-R2. No amplified product could be detected by agarose gel electrophoresis. Attempts at DNA sequencing using the combinations of primers utilized for PCR experiments seemed to confirm the absence of the gene encoding CbnB2 in *C. piscicola* 307 and 682.

4. Purification of BrcA and BrcB in *C. piscicola* using modified CCA medium

Cultures of pJKM56 (BrcA) and pJKM46 (BrcB) in *C. piscicola* LV17C (1% inoculum) were grown in a sterile modified [no Tween 80, erythromycin (5 μg/mL)] semidefined CAA medium[329] containing (per liter of solution): Casamino Acids (Difco Laboratories, Detroit, Mich.), 15 g; Bacto™ yeast extract (Difco Laboratories, Detroit, Mich.), 5 g; *D*-glucose, 20 g; dipotassium phosphate, 2 g; diammonium citrate, 2 g; magnesium sulfate, 0.1 g; and manganous sulfate, 0.05 g, at 25 °C at a constant pH of 6.7 by addition of filter-sterilized (0.22 μm) 2 M NaOH using a Chemcadet controller (Cole-Parmer, Chicago, IL 60648). After 21 h of incubation, cells were removed from the culture broth by centrifugation (8000 rpm, 20 min, 4 °C). The supernatant was extracted twice for 20 min with *n*-BuOH (500 mL/L of culture each time) and the emulsion was broken by centrifugation (6000 rpm, 10 min, 4 °C, polypropylene centrifuge bottles). The *n*-BuOH was evaporated under reduced pressure at low temperature (< 30 °C). The residue was suspended in H_2O (10 mL/L of culture), and extracted thrice for 20 min with $CHCl_3$ (150 mL/L of culture each time). The organic layer containing the desired bacteriocin was separated by centrifugation (2000 rpm, 4 min, 4 °C, Teflon centrifuge bottles) and evaporated under reduced pressure at low temperature (< 30 °C). The residue was suspended in a 1:1/MeOH:$CHCl_3$ mixture, and loaded at 4 °C onto a Sephadex LH-20 (Amersham Pharmacia Biotech, Québec, Canada) column (2.5 x 115 cm) and eluted at 0.5 mL/min (4 min/tube) with a 1:1/ MeOH:$CHCl_3$ mixture. All fractions collected were tested for antimicrobial activity by the spot-on-lawn assay, overlaying with LV17C(pMG36e). All pure active fractions were combined and analyzed by mass spectrometry, amino acid sequencing and circular dichroism.

5. Bacteriocin detection and activity assay

After each purification step, the arbitrary activity units of bacteriocin per milliliter (AU/mL) of concentrated solution was determined by taking the reciprocal of the highest dilution that exhibited a zone of growth inhibition showing extracellular complementation of BrcA and BrcB peptides. Fresh overnight cultures of pJKM46 and pJKM56 were spotted onto different APT agar plates supplemented with erythromycin (5 μg/mL) and incubated for 18 h at 25 °C. Beside the resulting colonies, the bacteriocins to be tested were spotted (10 μL), and an overlay of soft APT agar (6 mL) containing erythromycin (5 μg/mL) and an overnight culture of the indicator strain LV17C(pMG36e) (60 μL) was poured onto the plates. Plates were again incubated at 25 °C for 18 h and examined for clear zones of inhibition.

6. Purification of BrcA and BrcB in *C. piscicola* using complex peptone medium[336]

Cultures of pJKM56 (BrcA) and pJKM46 (BrcB) in *C. piscicola* LV17C (1% inoculum) were grown in a sterile complex peptone[336] medium [erythromycin (5 μg/mL)] containing (per 100 mL of solution): peptone, 1 g; *D*-glucose, 0.95 g; dipotassium phosphate, 0.2 g; magnesium sulfate, 10 mg; and manganous sulfate, 5 mg. Purification was achieved as previously described (Section 4 of Genetic Manipulations and Biological Experiments).

Preparation of unlabeled peptone: A culture of the cyanobacterium *Anabaena* sp. ATCC 27899 served as a producer for the peptone in a modified BG-11 medium containing (per liter of solution): $MgSO_4 \bullet 7H_2O$, 75 mg; $CaCl_2 \bullet 2H_2O$, 50 mg; $K_2HPO_4 \bullet 3H_2O$, 40 mg; Sea Water Mix (Bio-Crystals Marine-mix, Marine Enterprises, Baltimore, MD), 125 mg; ASTM Micro Elements Solution, 1 mL ($FeCl_3 \bullet 6H_2O$, 0.54 mg; Na_2-EDTA, 3 mg, H_3BO_3, 0.62 mg; $MnCl_2 \bullet 4H_2O$, 1.4 mg; $ZnCl_2$, 0.1 mg; MoO_3, 12 μg; $CoCl_2 \bullet 2H_2O$, 34 ng). The pH of the medium was adjusted to 7.6 prior to autoclaving at 121 °C for 20 min. The *Anabaena* sp. was maintained in 20 mL screw cap culture tubes (partially open to allow gas exchange) containing 10 mL of medium, gently swirled everyday, and reinoculated in fresh medium every 3 weeks. About 20 mL of the growing culture was transferred to a 2 L Erlenmeyer flask containing 500 mL of BG-11 medium. The standing cultures were propagated at rt under cool white fluorescent tubes for 12 days with gentle swirling twice per day. 1 L of cyanobacterium *Anabaena* sp. was centrifuged (15 min, 8000 rpm), the supernatant was removed, and the algae were used as an inoculum for an 8 L scale fermentation. This large scale fermentation was performed in a 10-liter glass vessel equipped with a magnetic stirrer, a temperature controller, a light source, a pH controller, and a fritted gas inlet. The pH of the gently stirred culture was maintained at 7.7 by the addition of 1 N HCl using a pH controller, and the temperature was kept at 28 °C. The oxygen produced by *Anabaena* sp. photosynthesis was removed with a continuous flow of argon. The argon was purified using CO_2-absorbing Ascarite, and was bubbled through the culture such that concentration of oxygen did not exceed 5-10% in the gas leaving the system. Solutions of $NaHCO_3$ in H_2O were continually added to the culture with a peristaltic pump (~0.9 mL/min) by passing through a sterile syringe filter (0.2 μm). The concentration of $NaHCO_3$ was increased during the growth process (1 g/60 mL for days 1-2, 2 g/60 mL for days 3-4, and 3 g/60 mL for days 5-11). $NaNO_3$ (1.5 g/10 mL H_2O) was added to the culture on day 1, 4, and 7 using a peristaltic pump (~0.9 mL/min) by passing through a sterile syringe filter (0.2 μm). Good growth was established by supplying light from 4 cool white fluorescent tubes surrounding the vessel and one filament tube located in the interior. After 11 days the cells were collected by centrifugation (8000 rpm, 20 min, 4 °C) and lyophilized, yielding ~0.8-1 g of dry cells per liter of fermentation. The dry cells were extracted with EtOAc in a Soxhlet extractor for 1 day to remove lipids, chlorophyll and other pigments. The resulting blue residue was resuspended in H_2O (1.5 g/100 mL) and digested at 37 °C with pepsin (Sigma, 3300 units/mg) at a concentration of 70 units/mL for 12 h at pH 2.0 (adjusted with concentrated HCl). Subsequently the pH was adjusted to 6.7 using 2 N NaOH, and the mixture was incubated with chymopapain (Sigma, 4.5 units/mg) at a concentration of 1 unit/mL for 36 h at 37 °C. The mixture was autoclaved (20 min, 121 °C) and the insoluble components were removed by centrifugation (8000 rpm, 15 min, 4 °C). The pellets were extracted with H_2O (2x300 mL) and centrifuged (8000 rpm, 15 min, 4 °C). All supernatant fluids were combined and lyophilized to yield ~5 g of peptone.

7. Purification of BrcA in *C. piscicola* using Celtone®-U complete medium

A 1-liter culture of pJKM56 (BrcA) in *C. piscicola* LV17C (1% inoculum) was grown in a sterile commercially available Celtone®-U complete medium (5013L0) (Spectra Stable Isotopes, Columbia, MD, USA) [erythromycin (5 μg/mL)] and purified as described previously (Section 4 of Genetic Manipulations and Biological Experiments).

8. Purification of BrcA in *C. piscicola* using SASM defined medium[338,339]

A 1-liter culture of pJKM56 (BrcA) in *C. piscicola* LV17C (1% inoculum) was grown in a sterile SASM defined medium [erythromycin (5 μg/mL)] and purified as previously described (Section 4 of Genetic Manipulations and Biological Experiments). The SASM defined medium[338,339] contains (per liter of solution): *D*-glucose, 10 g; 1 g each of $MgSO_4 \cdot 7H_2O$, $K_2HPO_4 \cdot 3H_2O$, KH_2PO_4, and $(NH_4)_2SO_4$; 10 mg each of $MnSO_4 \cdot H_2O$, $FeSO_4 \cdot H_2O$, and NaCl; 5 mg each of adenine, cytosine, guanine, uracil, and xanthine; 2 mg each of calcium pantothenate, thiamine hydrochloride, and niacin; 1 mg each of pyridoxine hydrochloride, riboflavin, inositol, $CuSO_4 \cdot 5H_2O$, and $ZnSO_4 \cdot 7H_2O$; 0.1 mg each of biotin and folic acid; and 0.1 g of all common amino acids. The pH was adjusted to 7.0, and the medium was filter-sterilized by passage through a 0.2 μm membrane filter.

9. Construction of expression clone of BrcA in *E. coli* (pSG1)

An expression clone of BrcA (pSG1) was constructed in *E. coli* BL21(DE3). To construct plasmid pSG1 (Figure 23), DNA from *E. coli* pT712 and pJKM61 was first isolated on large scale (Section 3 of the General Methods). The *dvn::brcA* gene, 292-bp fragment, from pJKM61 was then excised and ligated into the *Xba*I and *Sal*I sites of pT712. After transformation in competent cells of *E. coli* BL21(DE3) (Sections 5 and 6 of the General Methods), screening for the desired clone was performed (Section 8 ***Method A*** of the General Methods). Isolation of the DNA of the desired clone (pSG1) on large scale (Section 3 of the General Methods) was then done. DNA sequencing using primer BrcA rev confirmed the absence of mutations in construct pSG1 containing the gene encoding BrcA.

10. Construction of expression clone of BrcB in *E. coli* (pSG15)

An expression clone of BrcB was constructed in *E. coli* BL21(DE3). To construct plasmid pSG15 (Figure 23), DNA from *E. coli* pT712 was first isolated on large scale (Section 3 of the General Methods). DNA from *C. piscicola* pJKM46 was then isolated on large scale (Section 4 of the General Methods) using APT broth [erythromycin (5 μg/mL)] (50 mL) at 25 °C without shaking. The primer 71 (5'-ATATTCTAGATTGGAGGTTGGTATATATG-3') is based on the 5' end of the nucleotide sequence encoding the divergicin A signal peptide[276] and contains a *Xba*I restriction site (underlined). The reverse primer 72 (5'-ATATGAATTCGTATAGTTTTTACCATTGAT-3') is based on the 3' end of BrcB and contains an *Eco*RI restriction site (underlined). These two primers were designed to amplify the *dvn::brcB* gene fusion from pJKM46. Using the *Xba*I and *Eco*RI sites, the resulting PCR fragment was ligated into plasmid pT712 to give pSG15 (Figure 23). Taqplus precision polymerase was used to perform DNA amplification by PCR experiments (denaturation, annealing, and extension temperatures (1 min each): 94, 50, and 74 °C, 25 cycles). After transformation in competent cells of *E. coli* BL21(DE3) (Sections 5 and 6 of the General Methods), screening for the desired clone was performed (Section 8 ***Method A*** of the General Methods). Isolation of the DNA of the desired clone (pSG15) on large scale (Section 3 of the General Methods) was then done. DNA sequencing using primer APO9 confirmed the absence of mutations in construct pSG15 containing the gene encoding BrcB.

11. T7 RNA polymerase-directed expression of BrcA and BrcB in *E. coli*

Cultures of pSG1 and pSG15 in *E. coli* BL21(DE3) (1% inoculum) were grown (2 h) in LB broth [ampicillin (150 μg/mL)] with shaking at 37 °C to an optical density of 0.3 at 600 nm. They were subsequently induced for expression of the T7 RNA polymerase gene by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG; final concentration: 0.4 mM). After 1 h of incubation at 37 °C, rifampicin was added to a final concentration of 200 μg/mL. After an additional incubation of 1 h, cells were harvested by centrifugation (8000 rpm, 15 min, 4 °C) using a Sorvall RC5B centrifuge with a rotor model SLA-1500. They were washed and concentrated 100-fold in deionized water or ethanol, then lysed by French press at 4 °C. *n*-BuOH extraction (700 mL *n*-BuOH/1 L culture) of the supernatant, the French pressed cells, and the cell debris was followed by high performance liquid chromatography (HPLC). Both peptides were tested for activity on APT agar plates against *Carnobacterium piscicola* LV17C containing pMG36e (LV17C(pMG36e)) after each purification step.

12. High performance liquid chromatography

Purification of the peptides produced by pSG1 and pSG15 was achieved by HPLC using a reversed-phase diphenyl column (VYDAC 219TP54, 300 Å, 5 μm, 4.6 x 250 mm) on an analytical Beckman System Gold equipped with 32karat software. All compounds were detected at 218 nm, and eluted at 1 mL/min [X: H_2O (0.075% TFA), Y: MeOH (0.075% TFA)] using the following method: 10% Y for 2 min; increase to 70% Y in 2 min; increase to 100% Y in 8 min; stay at 100% Y for 13 min; decrease to 10% Y in 2 min; stay at 10% Y for 10 min. All fractions were tested for antimicrobial activity and analyzed by mass spectrometry.

13. Construction of MBP fusion of BrcA in *E. coli* (pSG618422)

A MBP fusion of BrcA was constructed in *E. coli* TB1. To construct plasmid pSG618422, DNA from *C. piscicola* pJKM56 was first isolated on large scale (Section 4 of the General Methods). The primer SG61351 (5'-TACAGTTCAAAAGATTGTCTA-3') is based on the 5' end of BrcA. The reverse primer SG61334 (5'-ATATGTCGACCTAGTTACCTAATAATCCACC-3') is based on the 3' end of BrcA and is complementary to the *Sal*I site (underlined) of pMAL™-p2X. These two primers were designed to amplify the *brcA* gene from pJKM56. Using the *Sal*I and *Xmn*I sites, the resulting PCR fragment was ligated at 0 °C overnight into plasmid pMAL™-p2X to give pSG618422. Platinum® *Taq* DNA Polymerase High Fidelity was used to achieve DNA amplification by PCR experiments (denaturation, annealing, and extension temperatures (1 min each): 94, 52, and 68 °C, 25 cycles). After transformation in competent cells of *E. coli* TB1 (Sections 5 and 6 of the General Methods), the *E. coli* transformants (100 μL/plate) were grown at 37 °C for 36 h on LB agar with a selective concentration of ampicillin (100 μg/mL). Preliminary screening for the desired clone was done by α-complementation using the blue-white selection method. Colonies were picked with sterile toothpicks, stabbed onto a master LB agar plate [ampicillin (100 μg/mL)] and onto a LB agar plate [ampicillin (150 μg/mL), Xgal (80 μg/mL), IPTG (0.1 mM)], and incubated at 37 °C for 36 h. The Lac phenotype on the Xgal plate was determined and the "white" clones were recovered from the corresponding patch on the master LB agar plate. A control transformation with about 1 ng of uncut pMAL^{TM}-p2X vector was performed to differentiate the various shades of blue colonies. Screening on the recovered "white" colonies was then performed (Section 8 ***Method A*** of the General Methods). After DNA isolation of each clone, digestion was first performed using *Sal*I. The digested DNA was then isolated and treated *Sac*I. Agarose gel allowed identification of the desired clone (pSG618422). The *brcA* gene was amplify by PCR experiments (denaturation, annealing, and extension temperatures (1 min each): 94, 52, and 68 °C, 25 cycles) to confirm its presence in the selected clone. Isolation of the DNA of pSG618422 on large scale (Section 3 of the General Methods) was done. DNA sequencing using primers SG61351 and SG61334 confirmed the absence of mutations in construct pSG618422 containing the gene encoding BrcA fused to a MBP.

14. Construction of intein protein fusion of BrcB in *E. coli* (pSG619685)

An intein protein fusion of BrcB was constructed in *E. coli* ER2566. To construct plasmid pSG619685, DNA from *C. piscicola* pJKM46 was first isolated on large scale (Section 4 of the General Methods). The primer SG61342 (5'-CCCAGGTTGTTGTACAGAACAAAATAAATTGGGGAAATGTTG-3') is based on the 5' end of BrcB and is complementary to nucleotides 6502-6483 of pTYB11. The reverse primer SG61343 (5'-ATATGTCGACTTACCATTGATCCCAAATACT-3') is based on the 3' end of BrcB and is complementary to the *Sal*I site (underlined) of pTYB11. These two primers were designed to amplify the *brcB* gene from pJKM46. The primer SG61331 (5'-TTCCCCTCTAGAAATAATTTT-3') is complementary to the *Xba*I site (underlined) of pTYB11. The reverse primer SG61341 (5'-CAACATTTCCCCAATTTATTTTGTTCTGTACAACAACCTGGG-3') is complementary to nucleotides 6502-6483 of pTYB11 and the forward BrcB nucleotide sequence. These two primers were designed to amplify the *intein* gene from pTYB11. Platinum® *Taq* DNA Polymerase High Fidelity was used to achieve DNA amplification of the *brcB* and *intein* genes by PCR experiments (denaturation, annealing, and extension temperatures (1 min each): 94, 52, and 68 °C, 25 cycles). The resulting PCR fragments were combined and amplified as a fusion system without primers using the following PCR experiments (denaturation:annealing:extension temperatures/94:52:68 °C/1:1:2 min, 5 cycles). Primers SG61331 and SG61343 were then added to the PCR mixture and the fusion system was amplified (denaturation:annealing:extension temperatures/94:52:68 °C/1:1:2 min, 25 cycles). Two bands were observed on agarose gel. The band corresponding to the fusion system was cut from the agarose gel and the DNA was separated from the agarose by gel electrophoresis performed in a dialysis bag filled with 0.5 TBE buffer at 170 V for 20 min. Purification of the DNA thus obtained was done by phenol extraction (200 μL), $CHCl_3$:isoamyl alcohol/24:1 extraction (200 μL), and DNA precipitation (EtOH 96%: NaOAc 3 M pH 5.0/400:25 μL, 12-16 h, 4 °C). Isolation of pure DNA was accomplished by centrifugation (12000 rpm, 30 min, 4 °C) followed by gentle wash with cold (4 °C) 70% EtOH (2x500 μL) and drying under vacuum (med rate, rotor on, 5-10 min) of the pure DNA pellets which were then dissolved in sterile TE buffer (20 μL) for 10 min at rt and kept at -20 °C. DNA amplification of the fusion system containing the *brcB* and *intein* genes was done by PCR experiments (denaturation, annealing, and extension temperatures (1 min each): 94, 58, and 68 °C, 35 cycles). Using the *Xba*I and *Sal*I sites, the resulting PCR fragment was ligated into plasmid pTYB11 to give pSG619685. After transformation in competent cells of *E. coli* ER2566 (Sections 5 and 6 of the General Methods), the *E. coli* transformants (100 μL/plate) were grown at 37 °C for 20 h on LB agar with a selective concentration of ampicillin (100 μg/mL). Preliminary screening on all the 88 colonies produced was then performed (Section 8 ***Method B*** of the General Methods). During screening, the PCR experiments were conducted as follow: (denaturation, annealing, and extension temperatures (1 min each): 94, 52, and 68 °C, 25 cycles). 19 out of the 88 clones were found to give PCR products. The DNA of 9 of these 19 clones was isolated (Section 8 ***Method A*** *Step 1* of the General Methods). Agarose gel allowed identification of the desired clone (pSG619685). Isolation of the DNA of pSG619685 on large scale (Section 3 of the General Methods) was done. DNA sequencing using primers SG61342, SG61343, SG61331 and SG61341 confirmed the absence of mutations in construct pSG619685 containing the gene encoding BrcB fused to an intein.

CHAPTER 4. References

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